University of Alberta

Salinity interactions with boron, root hypoxia and naphthenic acids in jack pine (*Pinus banksiana* Lamb.) seedlings

by

KENT GAZAL APOSTOL

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Salinity and its interactions with boron, hypoxia and naphthenic acids in jack pine (*Pinus banksiana* Lamb.) seedlings" submitted by Kent Gazal Apostol in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Forest Biology and Management.

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09 April 2003

DEDICATION

- For the greater glory of Almighty God, who is the source of strength and wisdom -

This work is dedicated to my beloved mother for her guidance, support and love throughout my graduate studies. Gone but always in remembrance, this thesis is dedicated in loving memory of my father, who had always encouraged and inspired me in his lifetime.

ABSTRACT

Open-pit mining of oil sands in north-eastern Alberta produces large volume of saline tailings. Aside from tailings being saline, they have high levels of boron (B), naphthenates and low levels of oxygen. These stresses can further exacerbate toxic effects of salt on plants and, consequently, affect the successful revegetation of the oil sands mining areas. The general objective of the present study was to understand the mechanisms of salinity interactions with other stress factors namely, B, root hypoxia and naphthenic acids (NAs).

To address the objective, jack pine (*Pinus banksiana*) seedlings were grown under controlled environment conditions and subjected to a combination of stresses, with salinity as a common stress factor. Results of the present work demonstrated the sensitivity of jack pine to salts and its susceptibility to salts was further increased by the presence of high concentrations of B, NAs and low levels of oxygen. Salinity altered jack pine responses to B. When present together with salts, B decreased survival and induced injury to plants. Among the distinct responses of jack pine to combined stresses were reduced root hydraulic conductance and stomatal conductance, which could alter root to shoot salt transport. The reduction in root water uptake observed in plants treated with NAs + NaCl explain the reduced accumulation of Na^+ and Cl^- in the shoots. In another experiment, I observed that under hypoxic conditions, roots lost the ability to restrict Cl^- uptake and the increase in Cl^- concentrations was correlated with root electrolyte leakage suggesting that Cl^- was partly responsible for membrane leakiness in the roots. Similar

response was also observed in plants exposed to B + NaCl treatments, which showed that the Cl^--induced membrane injury was partly responsible for Na^+ and B toxicity.

The implication of this research for the revegetation of the oil sands following mining operations is discussed.

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List of abbreviations, acronyms and symbols

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
B	Boron
BDL	Below detection level
CH_2Cl_2	Methylene chloride
Cl^-	Chloride
CT	Consolidated/Composite tailings
DMRT	Duncan's Multiple Range Test
DW	Dry weight
EC	Electrical conductivity
FT	Fine tailings
FT-IR Analysis	Fourier Transform Infrared Analysis
FW	Fresh weight
GLM	General Linear Model
g_s	Stomatal conductance
H_3BO_3	Boric acid
$HCO3^-$	Bicarbonate
HNO_3	Nitric acid
ICP-OES	Inductively Coupled Plasma Emission Spectroscopy
K_r	Root hydraulic conductance
L_p	Root hydraulic conductivity
Mpa	Megapascal
Na^+	Sodium
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NAs	Naphthenic acids
NEL	Needle electrolyte leakage
PPFD	Photosynthetic Photon Flux Density
Q_v	Steady-state root water flow rate
REL	Root electrolyte leakage
SE	Standard error

CHAPTER ONE

Introduction

In nature, plants are exposed to a variety of environmental stresses that can affect their physiological functions and cause injury. Among these stresses, salinity has been considered as one of the major soil factors inhibiting plant growth. Salinization occurs throughout the world, typically in areas with arid and semi-arid climates or in coastal regions, where salts are transported by groundwater and precipitation (Dudley 1994). Salts accumulate in the root zone as the soil water is used by plants for transpiration or lost by evaporation from the soil surface (Qadir et al. 2000). Such salt-affected soils develop as a result of natural-soil forming processes. Salt-affected soils also develop as a result of human activities through irrigation, overgrazing, deforestation and mining activities (Szabolcs 1994).

Open-pit mining results in severe land disturbance and often increases salt levels in the root zone following revegetation. Open-pit mining of oil sands in north-eastern Alberta has been a major contributor of petroleum produced in Canada since 1967. The Athabasca oil sands deposits contain an estimated 625 billion barrels of heavy crude oil (bitumen). Syncrude Canada Ltd., and Suncor Energy Inc. are the two major oil sands companies that are currently mining the Athabascan oil sands reserve. However, more development is planned for the area. Bitumen extraction uses a combination of hot water, steam and caustic soda (NaOH), which generates large volumes of tailings. The principal components of these tailings are aqueous suspensions of sand, silt, clay and residual bitumen. These tailings are discharged into large settling basins (Fung and Macyk 2000).

The predominant ions of fine tailings (suspended silt and clays) are sodium, chloride and sulfate. Other potentially phytotoxic components of tailings include boron and naphthenates (Herman et al. 1994). To reduce the volume of fine tailings, a new method of tailings management is being developed by Syncrude and Suncor. This technique, called Consolidated or Composite Tailings (CT) process, uses gypsum to precipitate the fine suspended materials and produces non-segregating CT deposit and release water (CT water) (Mikula et al. 1996). The high gypsum dosages that are required for this process result in a significant addition of ions (SO_4^{2-} from the gypsum, and Na^+ from Ca^{2+} exchange of clays).

Planned reclamation using CT involves a process known as dry capping which involves salvaging of suitable surface organic and mineral soils to be used for capping CT deposit followed by the establishment of self-sustaining vegetative cover. (Moneco 1983; Fung and Macyk 2000). Aside from salinity, a problem that has been noted is the development of oxygen deficiency (hypoxia) in the oil sands reclamation sites. Hypoxic conditions can occur due to high water levels and diffusion of methane, produced during anaerobic degradation of residual bitumen, from the tailings deposit that is present below the root zone (Fedorak et al. 2000). Under these conditions, methane may displace oxygen and produce temporary hypoxic conditions in the soil (Fedorak et al. 2000).

The results of the earlier studies suggest that salinity in the oil sands tailings materials (tailings water and tailings deposit) is the principal factor that inhibits growth of plants (Renault et al. 1998; 1999). Some effects of salt stress on plants are known, however, little work has been done to examine the responses of plants to salts in the presence of other stress factors such as those that are known to occur in the oil sands

reclamations areas including hypoxia, elevated levels of boron and naphthenates. It is possible that these stress factors can further exacerbate the phytotoxic effects of salinity and, consequently, affect successful revegetation of oil sands following mining operations.

Jack pine (*Pinus banksiana* Lamb.) is a small- to medium-sized coniferous tree of the northern forests in North America. A major portion of the jack pine range is in Canada where its northern boundary extends eastward from the Northwestern Territories to Nova Scotia. Jack pine grows in sandy soils, loams and soil over permafrost (Rudolf 1965; Cayford et al. 1967). It is considered to be a pioneer species on burnt and other exposed sites, and may persist and form an edaphic climax community on very poor and dry sites. Because of the ability of jack pine to grow on disturbed sites and its natural presence in the surrounding oil sands mining areas, it is considered a suitable species for reclaiming the oil sands mining areas. Other reasons for choosing jack pine for the present study were its commercial value for pulpwood, lumber and round timber (Cayford et al. 1967) and the legal restriction regarding the use of native species for reclamation (Fung and Macyk 2000).

The general objective of the present study was to understand the mechanisms of injury and tolerance of jack pine subjected to a combination of stress factors that are expected to affect oil sands reclamation plants. Understanding the growth and physiological responses of jack pine to these different stresses will assist oil sands and other mining industries as well as reclamation planners in developing and implementing successful strategies for the revegetation of mining areas. The focus of this research was

on growth and physiological processes in jack pine seedlings growing under controlled environmental conditions and subjected to different combination of stresses.

It has been reported that boron is a major factor contributing to the phytotoxicity of CT (Renault et al. 1998). It is, therefore, important to understand the mechanisms by which B affects growth and survival of jack pine. Several studies have been conducted to examine B uptake in plants, however, little is known about the effects of high B levels on water and nutrient uptake by plants. Since B accumulates in plants (Raven 1980; Dannel et al. 1998), its accumulation in plant tissues could alter nutrient balance and, in turn, result in injury and growth reduction in plants. Therefore, I examined growth, injury and root B and water uptake in jack pine seedlings. I also analyzed tissue elemental concentrations to test the hypothesis that B accumulation in plants decreases uptake of mineral nutrients and results in nutrient deficiencies (Chapter Three).

Plants may tolerate saline conditions through several mechanisms including osmotic adjustment, restriction of salt uptake and restriction of salt translocation to the shoots (Waisel 1991). However, these mechanisms may be affected by other environmental factors to which the plants are exposed to and which further aggravate salt-induced injury. Therefore, I conducted experiments to examine the effects of salinity in the presence of other potentially phytotoxic factors that have been identified in the CT.

Since relatively high levels of B are present in saline oil sands tailings, it is important to understand the interactions of B with salts and the resulting effects on plants. Renault et al. (1998) hypothesized that B may affect plant responses to salt. Therefore, in Chapter Four, I examined the combined effects of B and salts on growth, injury and ion composition in jack pine to test the hypothesis that B together with salinity causes greater

seedling mortality, needle injury and growth reductions compared with their individual effects alone.

Water deficits and ion uptake in salt-affected plants may also be affected by root hypoxia. Salinity tolerance is partly associated with the ability of plants to exclude salts from roots, and this process may be inhibited by hypoxic conditions. In Chapter Five, I tested the hypotheses that hypoxia interacts with NaCl by inhibiting water uptake and decreasing the ability of jack pine to restrict salt uptake.

The presence of naphthenic acids (NAs) in oil sands tailings may also hinder the growth and survival of seedlings planted in reclamation sites. Both NaCl (Carvajal et al. 1999) and NAs (Kamaluddin and Zwiazek 2002) upset water relations, which could affect Na^+ and Cl^- transport. Since NAs are chemically related to fatty acids (Brient et al. 1995), it is possible that they affect the functions of cell membranes. High concentrations of Na^+ and Cl^- in the shoots are often related to the reduced ability of plants to store these ions the roots (Yeo et al. 1977; Grieve and Walker 1983). The greater uptake of Na^+ and Cl^- is partly due to NaCl-induced root cell membrane injury (Kuiper 1968; Mansour 1997). In Chapter Six, I tested the hypothesis that NAs aggravate salinity effects by inducing salt accumulation in the shoots through their effects on cell membrane function and water uptake.

Finally, Chapter Seven summarizes the findings of all studies that were conducted and discusses the implications of this research for the reclamation of oil sands mining areas. In this final chapter, I also propose a conceptual model that explains how salt interactions with other stress factors affect the growh and physiological processes in jack pine.

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CHAPTER TWO

Literature Review

2.1. Distribution and biology of jack pine

Jack pine (*Pinus banksiana* Lamb.) is a small- to medium-sized northern coniferous tree native to the North America. A large part of the jack pine range is in Canada where its northern boundary extends eastward from the Northwestern Territories to Nova Scotia. This range extends south-west through Maine, central Quebec, and northern Ontario, north-east Illinois then north-west through Wisconsin, western Canada (central Alberta, Manitoba, Saskatchewan) to extreme north-east British Columbia (Rudolph and Yeatman 1982). Jack pine grows in maritime and continental climates with short, warm to cool summers, very cold winters and low rainfall. It is usually found on sandy soils and loams and soil over permafrost (Cayford et al. 1967; Rudolf 1965). It can also grow on calcareous soil with pH above 8 as long as mycorrhizal associations are present (Rudolph and Laidly 1990).

In the boreal forest, jack pine is associated with three forest cover types: black spruce type, paper birch type and aspen type. Jack pine is considered to be a pioneer species on burnt and other exposed sites. It may persist and form an edaphic climax community on poorest and driest sites, however, in the absence of fires and other catastrophic events, it may be succeeded by longer-lived species such as red pine (*Pinus resinosa*) or white pine (*Pinus strobus*) (Rudolf 1965).

Jack pine is a monoecious and wind-pollinated species. Fertilization in jack pine occurs about 13 months following pollination when the female cone is close to reaching

its maximum size (Ferguson 1904). Generally, jack pine bears serotinous cones which open most readily during dry weather when the temperature is at least 27°C. Some of the cones remain closed until they are exposed to fire or high temperatures of at least 50°C near the ground after wind breakage or logging (Rudolf 1965).

Normally, mature jack pine trees are about 17 to 20-m tall and 20-25-cm diameter at breast height (dbh). Rotation age of 40 to 50 years and 60 to 70 years are recommended to produce pulpwood and sawtimber, respectively (Rudolph and Laidly 1990). The wood is also used for telephone poles, fence posts, railroad ties and mine timbers (Alberta Department of Energy and Natural Resources 1977).

2.2. Oil sands reclamation concerns

Suncor Energy Inc. and Syncrude Canada Ltd., two large operating oil sands plants located in the Athabasca Oil Sands Deposit in northeastern Alberta (57°00'N 111°35' W), produce over 300 000 barrels of a sweet light crude oil per day from the extraction of over 500 000 tonnes of ore daily. This process uses hot water to separate bitumen from oil sand ore. However, the process also produces large volume of tailings. The biggest challenge is the disposal of these tailings (known as fine tailings, FT) since they slowly settle and tend to form a highly voluminous sludge (Marshall 1982). The predominant ions of FT include bicarbonate (HCO_3^-), sodium (Na^+), chloride (Cl^-), and sulfate (SO_4^{2-}). To reduce the volume of FT, the two commercial oil sands companies have developed a technique called the Consolidated or Composite Tailings (CT) process (Figure 2. 1). In this process, gypsum ($CaSO_4.2H_2O$) is added to the FT as a coagulant to speed up the consolidation process that would otherwise require hundreds of years to complete.

Consequently, ions leaching from the oil sands and chemicals added during processing increase ionic levels of CT. These CT deposits and CT water are relatively saline with electrical conductivity of > 4 mS cm^{-1} (MacKinnon et al. 2000). In CT, Na^+ and Cl^- are derived largely from ore and SO_4^{2-} is released from gypsum. The concentrations of these ions are expected to increase over time due to recycling of the process water. Increased salinity may affect successful reclamation of some parts of the boreal forest following mining operations. Aside from salts, phytotoxic levels of boron, fluoride and naphthenic acids (NAs) in the form of sodium salts (Lord and Nelson 1995) are also present in the CT water and they may interact with salts to cause further injury to plants.

In oil sands reclaimed areas, CT is deposited in large pits and capped with a layer of sand followed by a layer of reclamation material (salvaged muskeg and non-sodic overburden) (Moneco 1983). An additional challenge that has been observed is the possibility of oxygen deficiency (hypoxia) in the root zone of oil sands reclamation areas. Hypoxic conditions are likely to occur due to flooding of low-lying areas and diffusion of methane from tailings deposit, resulting in a depletion of soil oxygen (Fedorak et al. 2000). Since, the soil in low-lying areas is expected to become infiltrated with CT water and existing models suggest that salt will migrate to the surface (Feng and Sinha 1999), plants used in reclamation will have to cope with salinity under hypoxic conditions. Since these soil factors are known to be detrimental to plants, successful reclamation will depend on the ability of plants to tolerate salinity in the presence of other phytotoxic factors that may be present in the oil sands tailings.

2.3. Plant stress factors in the oil sands tailings

Plants are exposed to a range of chemical and physical factors which hinder their growth. In the oil sands, several of these factors are believed to be of major concerns to plants. Salinity is probably the predominant stress factor in the oil sands tailings. Together with boron, hypoxia and naphthenic acids, salinity is likely to affect the majority of oil sands reclamation areas.

2.3.1. Salinity

Salinity constitutes the most severe agricultural problem in many parts of the world. Salinization can occur in arid and semiarid areas or in coastal regions, where salts are transported by groundwater and precipitation. Salt deposition occurs by natural, physical and chemical processes as well as by human activities such as irrigation, land clearing and application of deicing salts to roads.

Salinity may result from an accumulation of different salts in the soil solution. However, much of the research that has examined salt effects on plants has been based on experiments in which sodium chloride was the predominant salt. This is because NaCl is particularly toxic and is the most common salt in many saline soils (Munns and Termaat 1986; Ashraf and Yousaf 1998; Rogers et al. 1998). Little research has addressed the effects of other common salts such as Na_2SO_4, KCl and K_2SO_4 (Warne et al. 1990; Bañuls and Primo-Millo 1992; Dudley 1994; Egan and Ungar 1998).

Salinity causes water deficit, specific-ion toxicity and nutritional disorders in plants and may result in injury and reduced growth (Cramer et al. 1985). Water deficit is

caused by a decrease in osmotic potential of the soil solution and by osmotic imbalance within a plant due to salt build up. When dissolved salt concentrations increase in the soil solution, water potential decreases, making it more difficult for water and nutrients to move through root membranes and into the plant. The end effects are similar to those of drought stress (Lambers et al. 1998). In addition to osmotic effects, there are ionic effects that arise from a specific composition of the solute flowing through the plant tissue. Reports suggest that Na^+ and Cl^- may alter cell membranes, and affect their function. Sodium has been reported to displace Ca^{2+} from the plasma membrane, followed by K^+ efflux (Cramer et al. 1985). Chloride has been found to alter membrane lipid composition resulting in increased membrane permeability (Kuiper 1968). These specific-ionic effects occur when ions accumulate to toxic levels in plants and begin to disrupt their metabolism directly. Internal excess of ions may cause membrane damage, interfere with solute balances or cause shifts in nutrient concentrations (Volkmar et al. 1998).

Salinity alters the nutritional balance of plants resulting in high ratios of Na^+/Ca^{2+}, and Na^+/K^+, which may cause reductions in growth (Cole 1985). High Na^+/Ca^{2+} and Na^+/K^+ ratios may impair the selectivity of the root membranes and result in a passive accumulation of Na^+ in the roots and shoots (Alam 1994). A high Na^+/K^+ ratio has been reported to decrease protein synthesis and enzyme activity (Botella et al. 1997). Grattan and Grieve (1992) showed that salt stress affects nutrient uptake through competitive interactions or by affecting the ion selectivity of membranes causing Ca^+ and K^+ deficiencies.

The degree to which the individual components of salinity stress influences plant growth is dependent on plant species, stage of plant development and ionic composition

of salts (Greenway and Munns 1980, Croser et al. 2001). Salt tolerance varies greatly among plant species and both survival and growth considerably change at different salinity levels (Storey 1995).

Glycophytes, which are less adapted to salts than halophytes, cannot retain high levels of salts in their tissues without suffering from salt injury. Halophytes, which typically grow in saline soils, preferentially accumulate salt in the leaves and this salt is used to balance the osmotic potential of the soil solution (Greenway and Munns 1980). Halophytes survive and grow in saline environments also because they are able to compartmentalize salt within the cells and this process partitions toxic ions away from the cytoplasm (Hasegawa et al. 2000). The main salt tolerance mechanism in glycophytes, is restriction of ion movement to the shoot by controlling ion influx to the root xylem (Volkmar et al. 1998).

The decline in leaf water potential and stomatal conductance observed in salt-treated plants was reported to be due to the osmotic effect on the roots affecting shoot water relations (Rodriguez et al. 1997). Recent studies also showed that NaCl decreased water channel activity and abundance affecting root hydraulic conductance (Carvajal et al. 1999; Carvajal et al. 2000). It has also been reported that the salt-induced decline in root hydraulic conductivity may be due to root growth reduction (Rodriguez et al. 1997). Some evidence suggests possible involvement of hormones such as abscisic acid in communicating soil water status to the shoots (Davies et al. 1986). ABA signals are involved in stomatal closure, and increases in ABA were related to restriction of Na and Cl accumulation in bean (Montero et al. 1997). Other studies showed that salinity

promotes senescence of plant tissues by increasing the production of ABA and ethylene (Kefu et al. 1991; Zhao et al. 1992).

Exposure of plants to salinity can either increase or decrease root respiration depending on the concentration of salts and on the duration of exposure. For example, root respiration of barley increased when exposed to 10 mM NaCl (Lambers et al. 1998). At moderate salinity (50 mM NaCl), Na concentrations in the roots increased while less Na accumulated in the shoots of oak (*Quercus robur*) seedlings (Epron et al. 1999). Prevention of Na translocation is probably linked to root respiration. According to Epron et al. (1999) the inability of oak to prevent Na translocation to the shoot was partly due to the salt-induced decline in root respiration.

Salt tolerance mechanisms include osmotic adjustment to allow cell water uptake under decreasing soil water potential, intracellular compartmentalization of salt and sequestration of salts in roots and stems to protect sensitive photosynthetic apparatus in shoots and production of stress proteins (Hagemeyer 1997). Although salt tolerance involves the integration of numerous physiological processes, there is considerable evidence that the ability of plants to exclude Na^+ and Cl^- from leaves is the most significant factor underlying intraspecific differences in salt tolerance. For example, tolerance of *Citrus* species can be determined by their capacity to exclude the potentially toxic Na^+ and Cl^- from stems and leaves (Storey 1995).

2.3.2. Boron

Boron is an essential micronutrient in higher plants (Hu and Brown 1997) and is required by plants in tissue concentrations of 5 to 15 ppm (Kozlowski and Pallardy 1997a). The optimum amount for one species could be either toxic or insufficient for another species (Blevins and Lukaszewski 1998). It has been reported that B is involved in enzyme activation, membrane maintenance, nucleic acid metabolism and sugar translocation and as an important constituent of the cell wall (Loomis and Durst 1992; Power and Woods 1997).

The highest concentrations of soil B are often found in marine evaporites or in marine argillaceous sediments (Erd 1980). High concentrations of B may occur naturally in soil, ground water, and areas adjacent to fly ash, chemical (glass, porcelain, domestic and industrial cleaning products) industries or surface mining areas (Nable et al. 1997).

Boron is absorbed by roots mainly as undissociated boric acid (H_3BO_3) and its uptake is primarily determined by B concentration in the external medium and plant transpiration rate (Hu and Brown 1997). Since boron transport in plants is influenced by transpiration (Raven 1980), leaves with high transpiration rates accumulate high B concentrations. Visible symptoms of B toxicity in leaves of sunflower plants occurred first at relatively high tissue concentrations prior to significant growth reduction (Dannel et al. 1998). Typical symptoms of B toxicity are chlorosis and necrosis at the tips and margins of the leaves (Eaton 1944). The degree of injury observed in leaves is directly related to leaf B concentrations (Grieve and Poss 2000). For some plants such as *Prunus*, stem death without accompanying chlorosis and necrosis of leaves is the visible symptom of B toxicity (El-Motaium et al. 1994).

At high B supply, B accumulates mostly in shoots (Wong et al. 1996). Plants tolerate B toxicity by restricting B uptake (Nable et al. 1997). Generally, the species that are tolerant of B are capable of maintaining low B concentrations in their shoots (Nable 1988; Paull et al. 1992) often by restricting transport to shoots (Nable et al. 1990). However, the exact mechanism of the restriction of B uptake is not well known. Many B toxicity studies have focused on visible toxicity symptoms and B accumulation, however, the mechanisms by which B accumulation in plant tissues affects nutrient uptake are still unclear.

2.3.3. Root oxygen deficiency (Hypoxia)

Oxygen deprivation, either complete (anoxia) or partial (hypoxia), is the main consequence of waterlogging or flooding of soils. Well-aerated solutions contain adequate soil oxygen ranging from 0.19 – 0.28 mol m^{-3} (6.0 – 8.8 mg O_2 l^{-1}) while some oxygen deficient soils contain 0.05 – 0.11 mol m^{-3} (1.5 – 3.5 mg O_2 l^{-1}) (Barrett-Lennard 1986). Oxygen deficiency in the rhizosphere may also be due to soil compaction which is common in agricultural and forested areas where heavy machinery is used. Hypoxia in waterlogged soils occurs because the diffusion coefficient of oxygen in water is about 10^4-fold lower than that in air (Grabble 1966). Flooding of soil can be due to overflowing rivers, storms, over-irrigation, inadequate drainage and impoundment of water by dams (Kozlowski and Pallardy 1997a). In western Canada, approximately 24, 000 ha were permanently waterlogged because of seepage from irrigation channels (Reid 1977).

Although shoot and root growth may be inhibited by hypoxia, roots are considered more sensitive than shoots (Huang et al. 1994). Similarly, Topa and

Cheeseman (1992) showed that root growth was affected more than shoot growth by oxygen deficiency stress in *Pinus serotina* Michx. Flooding may reduce water uptake and transport in plants (Everard and Drew 1989a) due to its inhibitory effects on root permeability and hydraulic conductivity (Everard and Drew 1989b). The decline in hydraulic conductivity of roots may also trigger stomatal closure resulting in reduced photosynthetic and transpiration rates (Drew 1983). Closure of stomates and reduced transpiration rates are among the early responses of plants subjected to flooding (Jackson et al. 1978; Bradford and Hsiao 1980).

Bradford (1983a) suggests a possible involvement of hormones in stomatal closure of plants exposed to hypoxia. Hypoxia increases endogenous abscissic acid (Setter et al. 1980). There are also several reports of ABA accumulation and concomitant reduction in stomatal conductance in leaves of flooded plants (Zhang and Davies 1987, Liu and Dickmann 1992; Zhang and Zhang 1994). It has also been reported that root hypoxia decreases cytokinin synthesis in roots (Bradford 1983b).

Under oxygen-deficient conditions, the energy pool decreases in roots, which, in turn, affects uptake of mineral nutrients. The uptake of nitrogen (N), potassium (K), phosphorus (P) and calcium (Ca) is often inhibited under flooding conditions, which reduces nutrient supply to shoots (Atwell and Steer 1990). A reduced level of K^+ was also shown to be partly responsible for stomatal closure in flooded pea plants (Zhang and Davies 1986). In addition to inefficiency of anaerobic respiration in providing adequate energy for active ion transport, the inhibition of nutrient uptake is also attributed to a reduction in root growth and increased permeability of cell membrane in roots (Drew

1983). The rapid efflux of K^+ from hypoxic roots was attributed to plasma membrane depolarization due to a decrease in ATP synthesis (Buwalda et al. 1988).

Flooding results in a lower concentration of ATP and the ratio of ATP to ADP decreases as a result of blocking of oxidative phosphorylation (Vartapetian 1991). Roots normally require oxygen for the optimal production of ATP. Under anaerobic conditions, only 2 molecules of ATP are produced from the oxidation of 1 molecule of glucose instead of 36 molecules of ATP in aerobic conditions (Morard and Silvestre 1996). The insufficient generation of ATP has important consequences for the metabolism of plants growing in oxygen-deficient conditions since ATP provides the energy for cellular processes (Drew 1983).

Flooding affects not only synthesis of carbohydrates, but also the transport of carbohydrates to meristematic sinks and their utilization in metabolism and production of new tissues (Kozlowski 1997). Root sugar content of some plants remained constant or increased when exposed to oxygen-deficient conditions (Barrett-Lennard et al. 1988). Setter et al. (1987) observed an increase in leaf carbohydrate concentration in flooded plants and a reduction in translocation of assimilates to roots of waterlogged plants (Jackson and Drew 1984). Daughtery and Musgrave (1994) suggested that the restricted availability of oxygen for root metabolic functions decreased the demand for assimilates resulting in an increase in leaf carbohydrate concentrations in *Brassica rapa*. On the other hand, Barclay and Crawford (1983) have shown that tolerance of plants to hypoxia is dependent on the available carbohydrate reserves in the roots. Waters et al. (1991) demonstrated that roots survived prolonged hypoxia when supplied with exogenous carbohydrates.

2.3.4. Naphthenic acids

Naphthenic acids (NAs) are complex mixtures of mono- and polycyclic alkanes containing carboxylated aliphatic side chains of various lengths. These hydrocarbons occur naturally within crude oils (Fan 1991, Seifert 1975). The structural formula of naphthenic acids is shown in (Figure 2. 2). NAs are often found as potassium, sodium and copper napthenates (Kaptyushina 1974; Shikina and Ovsyannikova 1974; Niyazov et al. 1977). Naphthenates are used in the wood preservation industry (Brient et al. 1995) and as emulsifying agents in the production of agricultural insecticides, in the oil industry as additives, and as emulsion breakers (Hatch and Matar 1977). Naphthenic acids in the oil sands tailings are present in the form of sodium salts and theier concentrations range from 50 – 100 mg l^{-1}. They are released from the bitumen during caustic hot water extraction process. It has been proposed that the presence of NAs is among the factors that likely contribute to the toxicity of the tailings waters to aquatic organisms (MacKinnon and Boerger 1986; Verbeek et al. 1993). However, there are also reports that shoot spraying of potassium naphthenates may stimulate seedling growth of conifer species (Wort and Kozak 1976). Other studies also revealed that soaking the seeds of *Phaseolus vulgaris* in potassium naphthenates stimulated IAA synthesis in the epicotyl tips (Loh 1974).

Despite the relatively high levels of NAs present in the tailings waters, their potential effects on plants have not yet been fully determined. Studies with aspen seedlings showed that NAs drastically reduced root water flow and stomatal conductance, which was partly due to the surfactant properties of NAs (Kamaluddin and Zwiazek 2002). It is, therefore, possible that NAs may interfere with water uptake and affect mineral nutrition of plants.

2.4. Interactions between salinity and other environmental factors in plants

Salinity negatively affects plant growth. The degree of salt injury may be affected by a number of environmental factors. Presence of factors such as boron, hypoxia and naphthenic acids in oil sands reclamation areas may aggravate the negative effects of salts in plants. However, the mechanisms by which various environmental stresses interact with salts are still poorly understood.

Salt is frequently not the only stress factor present in saline soils. High concentrations of B are often found in association with saline soils and saline well water (Dhankhar and Dahiya 1980; Gupta 1985). Although, salinity and B occur simultaneously, little is known of the responses of plants to excess B under saline conditions. Renault et al. (1998; 1999) demonstrated that salinity was not the sole factor responsible for sensitivity of boreal plants to CT. Due to potentially phytotoxic levels of B in CT, it is plausible that salinity-B interactions can occur in reclamation plants. Boron may have contributed to the injury observed in plants by interacting with salts and aggravating their injurious effects on plants.

Boron uptake is influenced by transpiration (Hu and Brown 1997). Plants exposed to saline conditions show reductions in transpiration rates due to the lowering of osmotic potential in the soil solution (Volkmar et al. 1998). Therefore, under saline conditions, B uptake and translocation might be inhibited by decreased transpiration rates. Marcar et al. (1999) showed that leaf B concentrations of *Eucalyptus* species decreased significantly with increasing salinity. This mechanism suggests that salinity may reduce the severity of B toxicity by lowering B accumulation in shoots (El-Motaium et al. 1994; Grattan et al. 1997). However, B concentrations in the leaves of *Cucumis sativus* continued to increase

despite the decreasing transpiration rates as a result of salinity (Alpaslan and Gunes 2001).

The literature suggests that combined effects of B and salts on plant growth could be species-dependent. For example, no significant interaction was observed for the measured growth parameters in *Eucalyptus camaldulensis* (Grattan et al.1997) and *Triticum aestivum* (Bingham et al. 1987) while significant interaction was seen in *Prunus* species (El-Motaium et al. 1994).

Biophysical and biochemical changes in plant cell membranes can be altered by a number of environmental factors. When membrane integrity is drastically altered, cell death follows shortly because of the loss of control of the cell transport processes (Mansour et al. 1993). Previous studies showed that NaCl caused injury to plants as a result of increased cell membrane leakiness (Mansour 1997). However, the possible contribution of B in aggravating the effects of salts on cell membrane integrity is poorly understood.

Oxygen deficiency may also occur simultaneously with salinity. Generally, saline oxygen deficiency conditions occur in coastal swamps and marshes and in poorly irrigated soils and low lying-areas subjected to primary and secondary salinization (Barrett-Lennard 1986). The interaction between hypoxia and salinity is known to affect adversely the growth of non-halophytes, however, the mechanisms involved in the interactive effects of salinity and hypoxia are not well understood. Salinity and hypoxia together caused greater reduction in growth and photosynthesis compared with their individual effects (Pezeshki 1992). Water deficits and excess ion accumulation are among

the factors that cause injury and growth reduction in plants exposed to saline and hypoxic conditions (Galloway and Davidson 1993).

Hypoxia is likely to interfere with the salt tolerance mechanisms that normally operate under well-aerated conditions. Some plants tolerate saline conditions by excluding ions from the xylem sap through energy dependent processes (Drew and Lauchli 1985). However, under hypoxic conditions, plants may be unable to prevent the transport of salts to leaves since less energy is available for ion exclusion (Barrett-Lennard et al. 1988; Drew and Lauchli 1985) resulting in salt accumulation in shoots. Naphthenic acids are natural components of crude oils (Fan 1991) but we know little about their effects on plants and there are no published reports on their interactions with salts in plants. It is possible that due to their surfactant properties, NAs might aggravate the salt stress effects on water uptake. Since NAs are chemically similar to fatty acids (Brient et al. 1995), it is plausible that NAs may affect cell membrane function and alter salt uptake.

2.5. Water and salt movement through plants

Roots absorb water from the soil in response to a water potential gradient. Water is transported from the root surface to the root xylem. There are three different pathways for the radial movement of water and solutes in roots. These are the apoplastic pathway (outside of the protoplasts), symplastic pathway (through the plasmodesmata), and transcellular pathway (across the membranes). Once water and dissolved solutes enter the xylem, they are transported to the shoot via the transpiration stream. The root water flow

is best described by a composite transport model, which explains the variability in root water uptake and responses of water uptake to different factors (Steudle 2000a).

Water flow through a root can be expressed by the root hydraulic conductance or root hydraulic conductivity. The general principle of measuring root hydraulic conductance (K_r) is similar to the measurement of membrane permeability. For a membrane, a water potential gradient is established across the membrane and the flow of water is measured. The conductance of the membrane is calculated using this equation: $Q_v = \Delta\psi_w L$ where Q_v is the volumetric flow rate of water ($cm^3\ s^{-1}$), $\Delta\psi_w$ is the water potential gradient (MPa), and L is the hydraulic conductance ($cm^3\ s^{-1}$ MPa) (Fiscus 1975). Conductance of the root is the inverse of the resistance; that is the slope of the ratio between water potential and water uptake. It is different from root hydraulic conductivity (L_p) since the total root surface area is not measured.

The ability of roots to transport water to the shoots is determined by the root hydraulic conductance (Kramer 1983; Huang and Nobel 1994). Water uptake by the roots is a variable process that depends on the species (Rieger and Litvin 1999), root structure (North and Nobel 1995), time of the day (Else et al. 1995; Clarkson et al. 2000), and root age (Barrowclough et al. 2000). It is also influenced by various environmental factors such as water deficit stress (Steudle 2000b), temperature (Wan et al. 1999), hypoxia (Kamaluddin and Zwiazek 2001), as well as salinity and ion toxicity (Carvajal et al. 2000).

Root hydraulic conductance may be inhibited by environmental factors through the effects on the activity of aquaporins (Carvajal et al. 2000). Aquaporins are protein molecules that form channels allowing water to pass freely across the cell membranes

(Chrispeels and Maurel 1994). Studies on aspen showed that water channels of cell membranes control root hydraulic conductivity and affect seedling water relations (Wan and Zwiazek 1999). Similar to root hydraulic conductivity, a decrease in stomatal conductance is also observed in plants exposed to environmental stresses (Wan et al. 1999; Carvajal et al. 2000; Steudle 2000b; Kamaluddin and Zwiazek 2001). The decrease in stomatal conductance has been shown to be the result of reduced root hydraulic conductivity in NaCl-treated tomato plants (Rodriguez et al. 1997).

Although not confirmed yet, Yeo (1998) suggests that movement of salt into roots and shoots is a product of the transpirational flux required to maintain the water balance in the plant. However, there are reports suggesting that salt uptake may involve active transport. Excessive transpiration can result in toxic levels of ions accumulating in the shoots (Hasegawa et al. 2000). Munns and Termaat (1986) suggested that stomatal closure is the immediate response of plants to salinity, which limits ion flux to the shoot.

2.6. Effects of salinity and CT-associated stress factors on conifers

Most salinity research to date has concentrated largely on agricultural crops and fewer studies have investigated the effects of salinity, boron and root hypoxia on woody plants, particularly conifers. Salinity reduced growth and stomatal conductance of *Pinus pinaster* (Loustau et al. 1995, Croser et al. 2001). Salinity also reduced emergence and seedling growth of *Picea glauca* (Maynard et al. 1997). Application of NaCl to foliage of *Picea glauca* caused the disruption of stomata, collapse of cell walls, and disintegration of chloroplasts and nuclei (Stewart et al. 1973). High concentrations of Na caused premature needle loss and chlorosis in *Pinus pinaster* (Nguyen-Queyrens et al. 1995), and growth

reduction of *Pinus radiata* (Sands and Clarke 1977) and *P. taeda* and *P. eliottii* (Land 1974) were attributed to foliar accumulation of Na and Cl ions.

Since, natural occurrence of boron toxicity in forest areas is not common (Stone 1968), little emphasis has been placed on the effects of B on trees. The presence of high levels of B in forested areas is a result of misapplication of B fertilizer and application of borax as herbicide (Stone 1990). High concentrations of B impaired root mass formation more strongly compared with needles in *Pinus sylvestris* (Fiedler et al. 1984) and induced needle necrosis due to collapse of mesophyll cells (Stewart et al. 1973).

Flooding inhibited shoot growth in *Pinus. echinata*, *P. taeda* and *P. serotina* (Hunt 1951), *Picea glauca, P. marina*, *Pinus banksiana*, *P. resinosa*, *P. strobus* (Ahlgren and Hansen 1957) and needle formation in *P. banksiana* and *P. resinosa* (Tang and Kozlowski 1983). Flooding also induced reductions in root growth of *P. banksiana* (Tang and Kozlowski 1983), *P. contorta* (Coutts 1982), *Picea mariana* and *Larix laricina* (Lieffers and Rothwell 1986). Some pine species appear to be particularly sensitive to flooding. For example, *P. banksiana* is considered more sensitive to flooding than *P. resinosa* (Ahlgren and Hansen 1957, Tang and Kozlowski 1983).

The mechanisms by which plants tolerate flooded conditions are complex and involve interactions of morphological, anatomical and physiological adaptations (Kozlowski and Pallardy 1997b). In a study by Conlin and Lieffers (1993), the authours observed that *Pinus contorta* and *Larix laricina* were better adapted to flooding than *Picea glauca and P. mariana* due to their ability to transport O_2 to root tissues and sustain limited respiration under oxygen-deficient conditions. Coutts and Philipson (1978) also reported that flooded *P. contorta* developed cavities in the stele that

facilitated air flow to submerged roots. Some species develop extensive adventitious root systems when stems are flooded, and these roots facilitate absorption of water and nutrients. For example, waterlogging induced formation of adventitious roots in flood-tolerant *P. contorta* and *Picea sitchensis* (Kozlowski 1984). Formations of hypertrophied lenticels, which facilitate exchange of dissolved gases in flood water, have been also reported for *Pinus banksiana*, *P. ponderosa*, *Picea mariana* and *Larix laricina* (Hahn et al 1920).

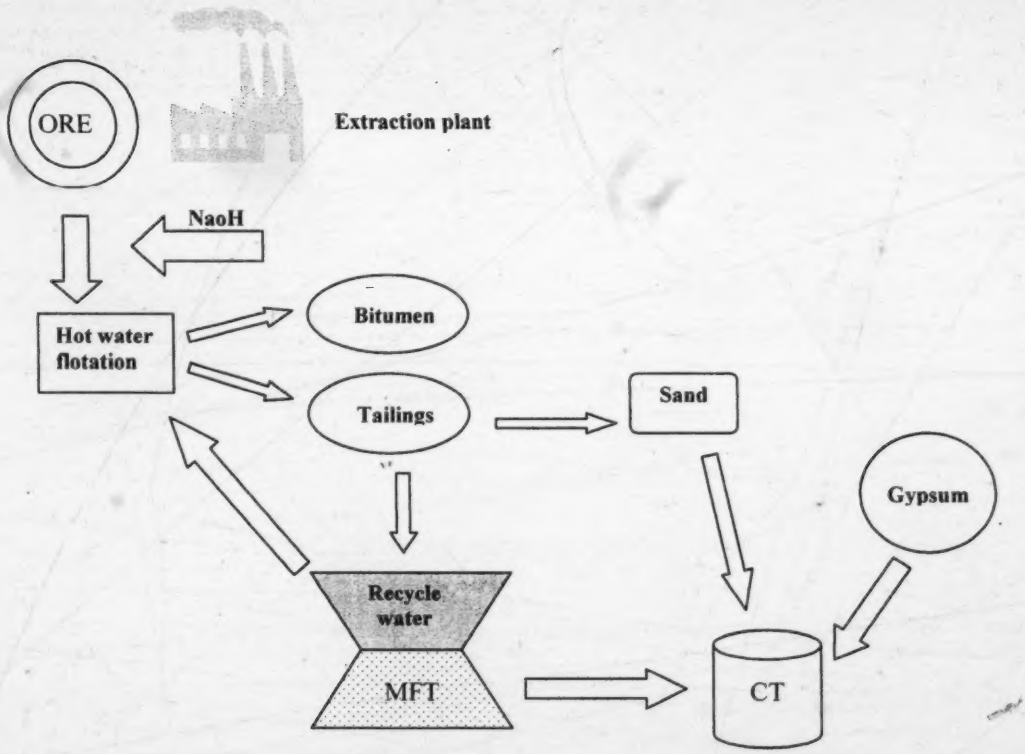

Figure 2. 1. Schematic diagram of Consolidated/Composite Tailings (CT) process. MFT - mature fine tailings.

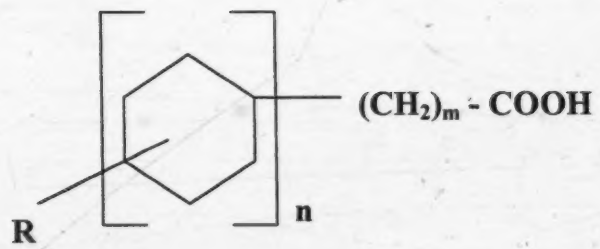

Figure 2. 2. A structural formula of naphthenic acids modified from the Encyclopedia of Chemical Technology (1981), in which m is greater than one, n can range from one to more than six, and R is a short aliphatic chain.

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CHAPTER THREE*

Boron and water uptake in jack pine (*Pinus banksiana*) seedlings

3.1. Introduction

Boron, which is widely distributed in lithosphere and hydrosphere (Power and Woods 1997), is an essential micronutrient in higher plants (Hu and Brown 1997). Boron requirements vary among species from 5 to 15 ppm (Kozlowski and Pallardy 1997). The optimum quantity for one species could be either toxic or insufficient for another species (Blevins and Lukaszewski 1998). Boron is an important constituent of the cell walls and has been reported to be involved in enzyme activation, membrane maintenance, nucleic acid metabolism and sugar translocation (Loomis and Durst 1992, Power and Woods 1997). Boron deficiency is commonly associated with acidic soils, and in sands low in silt and clays (Stone 1990). High concentrations of B are present in arid areas, and areas disturbed by surface mining, including oil sands in western Canada (Nable et al. 1997, Renault et al. 1998).

High concentrations of B may cause reductions in chlorophyll concentrations, CO_2 fixation and leaf areas leading to growth inhibition and injury in plants (Lovatt and Bates 1984, Nable et al. 1997). In conifer species, necrosis caused by high levels of B is thought to be due to collapse of mesophyll cells (Stewart et al. 1973). A typical symptom

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of B toxicity is characterized by the presence of chlorosis and necrosis, often at the margins and tips of older leaves (Eaton 1944). While several studies have been conducted to examine mechanisms of B uptake in plants (Raven 1980, Hu and Brown, 1997, Dannel et al. 1998), little emphasis has been placed on examining the effects of B on water and nutrient uptake by plants.

Transpiration is among the key factors likely to affect B uptake (Hu and Brown 1997). Since uptake of most ions is driven by transpirational pull of water, a reduction in transpiration rates could result in reduced uptake and transport of B to shoots. Water transport through the roots, which is believed to affect shoot water relations and ion uptake, is inhibited by several environmental factors including low temperature (Wan et al. 1999) salinity (Carvajal et al. 2000) and hypoxia (Kamaluddin and Zwiazek 2001). A decline in root hydraulic conductance may be caused by a reduction in root growth. Therefore, it is conceivable that root growth reduction observed in plants treated with excess boron (Dannel et al. 1998) could affect root water flow properties. Plants exposed to high B concentrations could show reductions in root water flow and water uptake, which, in turn, could affect nutrient uptake.

Since B accumulates in plants (Dannel et al. 1998, Raven 1980), this accumulation could also alter nutrient balance. Therefore, it is possible that growth reduction observed in B-treated plants (Stewart et al. 1973, Nable et al. 1997, Dannel et al. 1998) could be partly due to nutrient deficiency.

Jack pine (*Pinus banksiana* Lamb.), a tree species native to Canadian boreal forest, is an early successional species in sandy and nutrient-poor sites (Cayford et al. 1967). Because of these characteristics, jack pine is considered to be one of the candidate

species for the revegetation of sites disturbed by surface mining in northwestern Canada where B levels may be elevated in the soil. In the present study, I used jack pine to examine the mechanisms of B toxicity. I examined the hypothesis that B accumulation in plants leads to reduced water transport, which, in turn, decreases plant uptake of mineral nutrients and results in nutrient deficiencies.

3.2. Materials and methods

3.2.1. Plant material and growth conditions

One-year-old dormant *Pinus banksiana* seedlings were obtained from the Pine Ridge Forest Nursery in Smoky Lake, AB, Canada. The roots, which had been growing in a mixture of peat and perlite, were washed with deionized water and transplanted into 200-ml Spencer-Lemaire Root trainers filled with washed quartz/feldspar sand (Steuwe and Sons, Inc., Corvallis, OR, USA). Twelve 10-l containers, each with six seedlings arranged in 4-l pots, were placed in a growth room under the following environmental conditions: 18-h photoperiod, 22/18°C day/night temperature, 70% RH, and 250-300 μmol m^{-2} s^{-1} Photosynthethic Photon Flux Density (PPFD) at seedling height. The seedlings were fertilized with nutrient solution recommended for jack pine. The nutrient solution contains 80 mg l^{-1} N, 60 mg l^{-1} P, 104 mg l^{-1} K, 100 mg l^{-1} Ca, 60 mg l^{-1} Mg, 79 mg l^{-1} S, 3 mg l^{-1} Fe, 0.40 mg l^{-1} Mn, 0.25 mg l^{-1} B, 0.14 mg l^{-1} Zn, 0.50 mg l^{-1} Cu and 0.10 mg l^{-1} Mo (Wood 1995) and were grown for one week following transplanting before B treatments were initiated.

3.2.2. Boron treatments

Treatment solutions were prepared by mixing stock solution of B (H_3BO_3) with conifer nutrient solution until the desired concentrations of 0.5, 1 and 2 mM B concentrations were achieved. The conifer nutrient solution contained 1.43 mg l^{-1} of H_3BO_3, equivalent to 0.25 mg l^{-1} of B. I used concentrations somewhat higher than the present levels of B found in the oil sands tailings (Renault et al. 1998, Renault et al. 1999) to shorten the time of study. It is also expected that these concentrations will increase with time. Seedlings were treated with B by adding 3-l of treatment solutions into the 10-l containers. Conifer nutrient solution was also supplied to serve as a control. Control and treatment solutions were replaced every week and seedlings in pots were removed from the containers and placed on the bench every 24-h to freely drain the treatment solution from the bottom of the containers. The design of the study was randomized with three containers per treatment solution each containing six plants.

3.2.3. Survival and growth measurements

Survival was monitored daily for six weeks from the initiation of treatments until the harvest date. At harvest, 6 seedlings per treatment were randomly selected for new shoot length measurements. New roots ≥ 5 cm were counted and fresh weights of shoots and roots were determined.

3.2.4. Stomatal conductance (g_s) and steady-state flow rates (Q_v) measurements

Immediately prior to harvesting and between 3 and 4 h after the onset of the photoperiod, stomatal conductance (g_s) was measured on the upper 3-cm portion of the shoot using a steady-state porometer LI-600 (Li-Cor Inc., NE, USA). Needle area was measured using a scanner-based computer program (Sigma Scan 3.0, Jandel Scientific, San Rafael, CA, USA).

Measurements of steady-state flow rates (Q_v) were done using the hydrostatic pressure method (Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2001). A 0.25-l glass cylinder was placed inside the pressure chamber (PMS Instruments, Corvallis, OR, USA) and filled either with treatment or control bathing solution, which was continuously stirred with a magnetic stirrer throughout the measurements. A stem was severed above the root collar region and the entire root system was sealed in the pressure chamber. The whole root system was immersed in the bathing solution, 2 mM B + nutrient solution for treated plants and plain nutrient solution for control plants, with the debarked stem end protruding through a rubber gasket fitted in the lid of the pressure chamber. A pressure of 0.3 MPa was gradually applied and held constant throughout the measurements. A graduated pipette was fitted to the protruding stem by connecting the stem and the pipette with a short piece of rubber tubing. Flow rates of the entire root systems (Q_v) were recorded every five minutes for thirty minutes and the results were expressed in µl H_2O root system^{-1} min^{-1}.

3.2.5. Needle necrosis and electrolyte leakage measurements

Needles were separated into green (living tissues) and brown (necrotic tissues) portions to quantify the proportion of needle necrosis. Necrosis was expressed as a percentage of the total needle dry weight.

For the electrolyte leakage test, a method used to assess cell membrane integrity, six seedlings per treatment were randomly harvested and washed with deionized water three times, for five minutes each. The electrolyte leakage test is a method that detects the loss of electrolyte from semi-permeable cell membranes (Zwiazek and Shay, 1988) as described by Renault et al., (1998). Briefly, samples containing 0.45 g of needles were placed in tubes, each containing 10 ml of deionized water. After 1-h incubation, the solutions were replaced with another 20 ml of deonized water and incubated for 5 h at 50 RPM in an orbital shaker. Electrical conductivity of the solutions was measured with an electrical conductivity meter HI 8033 (Hanna Instruments Inc., Woonsocket, RI). Total electrolytes remaining in tissue were obtained by autoclaving the samples at 121 ^{o}C followed by freezing overnight at –85^{o}C. Electrolyte leakage was expressed as a percentage of total electrolytes.

3.2.6. Tissue elemental analysis

Plant tissues were freeze-dried, ground in liquid nitrogen to a size that would pass through a 2-mm sieve, and used for the elemental tissue analysis. Tissue elemental concentrations were determined using the Inductively Coupled Plasma Emission

Spectroscopy (ICP-OES) (Vista-RL, Varian Inc., Victoria, Australia) after digesting samples in 5% HNO_3 (Renault et al. 1999).

3.2.7. Statistical analysis

Analysis of variance (ANOVA), linear regression and correlation analyses were performed using SAS GLM (General Linear Model) (SAS Institute Inc., Cary, NC, USA). The means were compared using the Duncan's Multiple Range Test (DMRT), and were considered significantly different at $P \leq 0.05$. The model used was:

$$Y_{ij} = \mu + \rho_i + \tau_j + \varepsilon_{ij}$$

where:
Y_{ij} = response variable;
μ = overall mean;
ρ_i = replication (random factor), i = 1,...3;
τ_j = boron effect (fixed factor), j = 1,...4; and
ε_{ij} = random error

For the linear regression, needle necrosis and needle electrolyte leakage were separately used as the dependent variable and needle boron concentrations served as an independent variable. The formula was as follows:

$$Y_i = \alpha + \beta x_i + \varepsilon_i$$

where:
Y_i = dependent variable;
α = Y intercept of regression;
βx_i = coefficient of regression;
x_i = independent variable; and
ε_i = random error

3.3. Results

Boron did not significantly affect survival and measured growth traits of jack pine after six weeks of treatments (Table 3. 1). However, B significantly ($P \leq 0.0083$) reduced g_s (Figure 3. 1). Plants treated with the highest, 2 mM B concentration, showed almost 40 % reduction in g_s compared with the control plants. Results of the 4-week exposure of jack pine to 2 mM B also showed a significant ($P<0.0019$) decline in g_s (Figure 3. 2a) and a simultaneous inhibition of Q_v ($P<0.0269$) (Figure 3. 2b). Mean Q_V in seedlings treated with 2 mM B declined to about 40% of the rate measured in control seedlings.

Needle injury began to appear after two weeks of B treatments and was characterized by yellowing and browning of needle tips (Figure 3.3). The symptoms progressively developed towards the bases of the affected needles. Needle necrosis was first observed among the older needles (expanded before B treatments) and then gradually developed among the new needles (expanded during B treatments). Plants treated with 2 mM B exhibited severe needle necrosis, which constituted about 15% of the total needle dry weight (Figure 3. 4a). Needle electrolyte leakage significantly increased ($P \leq 0.0001$) in response to B treatments. Plants treated with 2 mM B showed 60% higher electrolyte leakage compared with control plants (Figure 3. 4b).

Boron concentration in both needles and roots increased as B concentration in the treatment solutions increased (Figure 3. 5.). Relatively more B accumulated in needles compared with roots. Plants treated with 2 mM B showed 67 % greater B concentration in needles than those in the roots.

Needle necrosis in jack pine increased significantly with increasing needle B concentrations (Figure 3. 6a). Similarly, needle electrolyte leakage increased with increasing B concentrations in needles (Figure 3. 6b).

Results of the tissue elemental analysis showed no significant difference in shoot macronutrient concentrations among B treatments (Table 3. 2). Micronutrients were present below the detection level.

3.4. Discussion

After six weeks of treatments, boron significantly increased cell membrane injury and needle necrosis in jack pine, however, needle injury observed in B-treated plants did not affect the measured growth parameters over the course of the treatments. Boron has been reported to reduce biomass, carotenoid and chlorophyll contents in buffalograss (*Buchloe dactyloides*) (Jackson et al. 1995). In some plants, toxicity symptoms developed following decreases in chlorophyll concentrations, growth and leaf areas (Lovatt and Bates 1984). However, similar to my results, B toxicity symptoms in sunflower leaves were observed prior to significant growth reductions (Dannel et al. 1998).

Jack pine accumulated B mostly in needles. Similar results were reported for sunflower with leaf B concentrations 10 times larger than in the roots (Dannel et al. 1998). In *Prunus* species, stem death, which was the pronounced observed symptom, was solely related to stem accumulation of B (El-Motaium et al. 1994). In this study, B did not appear to accumulate in stems and there were no detectable levels of B found in stems in any of the applied B treatments. Tolerance to high levels of B depends on the ability of the plants to preferentially distribute B at the cellular, tissue or organ level (Nable et al.

1997). Generally, species that are tolerant of B are capable of maintaining low B concentrations in their shoots (Nable 1988, Paull et al. 1992). The lack of growth inhibition in jack pine six weeks after treatment suggests that jack pine was able to keep B away from key metabolic sites.

Similarly to *Brassica napus* (Asad et al. 1997), increasing B concentrations in treatment solutions resulted in greater B concentrations in needles and roots, and higher needle necrosis (Figure 3. 4a). I have found a positive correlation ($r = 0.908$; $P \leq 0.05$) between needle necrosis and B concentration in the needles. My electrolyte leakage data also showed a link with B accumulation suggesting that B may produce direct effects on cell membranes before necrosis can be observed. Nable and Paul (1991) demonstrated that B accumulation in plants might be related to differences in membrane permeability associated with membrane and cell wall composition.

My results suggest that B did not significantly alter nutrient status of jack pine treated with B for six weeks (Table 3. 2). The lack of nutrient deficiency may also explain the absence of growth reduction. Although, I did not measure tissue N levels, I did not observe visible N deficiency symptoms or chlorophyll reductions (Table 3. 3). Nitrogen deficiency is known to result in chlorophyll reduction therefore, the absence of chlorophyll reductions observed in the present study suggest that tissue N concentrations were not significantly reduced in B-treated jack pine. Deficiency of an essential element drastically reduces growth (Masoni et al. 1996). In the present study, there was no specific nutrient deficiency causing needle injury which suggests that nutrient deficiency was not a factor contributing to needle death or necrosis.

Since B uptake is driven by transpirational pull of water (Kozlowski and Pallardy 1997), reduction in g_s, which is associated with a decline in transpiration rates, could lead to a decline in B uptake. In another study (Chapter Four), salts reduced B uptake due to their inhibitory effects on transpiration. In the present study, I related the reduction in g_s to the increasing B concentrations in needles. It is possible that increased B accumulation in needles induced cell dehydration leading to stomatal closure. Plants exposed to salt demonstrated partial stomatal closure when sodium in the apoplast around the guard cells began to rise (Perera et al. 1994), probably to avoid excessive tissue dehydration and reduce transport of ions to the shoots. I am not certain if the decline in g_s was a direct response of plants to B or a consequence of needle necrosis due to B accumulation in needles. In the longer term, the reduction in g_s could limit photosynthetic capacity and negatively affect growth rates.

Wan et al. (1999) showed that low root temperature caused a reduction in root water transport via root growth inhibition. In the present study, however, the decline in Q_v was not related to root growth since I did not observe root growth inhibition. My data showed that B reduced Q_v with a concomitant decline in g_s (Figure 3.2). Previous studies revealed that both g_s and Q_v were reduced when plants were exposed to different environmental stresses (Wan et al. 1999, Kamaluddin and Zwiazek 2002). It appears that the water conductance properties are affected in a synchronized manner. However, their relationship is not completely understood (Jackson et al. 1996, Clarkson et al. 2000). The exact mechanism by which B reduced Q_v is not clear. It is possible that the inhibition of Q_v could be triggered by stomatal closure, however, the hydraulic effects, which originates in the roots, on stomatal movements cannot be discounted.

In the present study, I demonstrated that B inhibited stomatal conductance, root water flow and caused needle injury in jack pine seedlings. However, B did not alter tissue nutrient status of jack pine, which suggests that nutrient status was not related to needle injury. Contrary to my hypothesis, the reduction in root water flow was not related to B accumulation and did not appear to affect the nutrient uptake and distribution in jack pine during the course of the treatments. From the data presented here, needle necrosis observed in B-treated plants was related to B accumulation. Further studies are necessary to verify the exact mechanisms of stomatal regulation, water uptake and B transport in jack pine.

Table 3. 1. Percent survival, new shoot length, number of new roots, shoot and root fresh weights of jack pine treated with boron. Plants were grown in sand culture for six weeks. Values represent means (SE), n=6. There was no significant difference between treatments at $P \leq 0.05$ based on Duncan's Multiple Range Test (DMRT).

Treatment	Percent survival	New shoot length (cm)	Number of new roots	Shoot fresh weight (g)	Root fresh weight (g)
Control	94.44 (1.73)	7.03 (0.81)	10.16 (2.02)	3.18 (1.01)	1.01 (0.46)
0.5 mM B	94.00 (1.73)	5.90 (1.08)	10.00 (2.99)	3.22 (0.55)	1.85 (0.39)
1.0 mM B	100.00 (0.00)	7.90 (1.25)	8.67 (1.60)	3.21 (0.12)	0.97 (0.28)
2.0 mM B	100.00 (0.00)	7.10 (0.60)	8.50 (1.35)	2.81 (0.51)	1.17 (0.32)

Table 3. 2. Tissue elemental concentrations (mg kg^{-1} DW) in jack pine treated with boron. Plants were grown in sand culture for six weeks. Values represent means (SE), n=6. There was no significant difference between treatments at $P \leq 0.05$ based on Duncan's Multiple Range Test (DMRT), BDL = below detection level.

Elements	Tissue	Treatment			
		Control	0.5 mM B	1.0 mM B	2.0 mM B
Ca	Needle	5972 (305)	5912 (505)	5756 (689)	6578 (218)
	Stem	3378 (309)	3086 (208)	2866 (129)	3115 (127)
	Root	9996 (452)	10680 (936)	9018 (760)	8806 (299)
K	Needle	6516 (601)	7244 (445)	6296 (601)	6560 (491)
	Stem	6594 (656)	7744 (377)	7840 (603)	7188 (774)
	Root	7864 (2026)	9880 (934)	8188 (456)	7238 (1470)
Mg	Needle	2574 (135)	2638 (303)	2514 (231)	2651 (90)
	Stem	1966 (223)	1660 (103)	1778 (141)	1651 (154)
	Root	2722 (266)	3030 (100)	2884 (92)	2601 (136)
P	Needle	2078 (122)	2046 (278)	1818 (77)	1910 (35)
	Stem	1420 (78)	1772 (190)	1570 (80)	1688 (189)
	Root	4184 (511)	4092 (273)	4066 (225)	4038 (175)
S	Needle	BDL	BDL	BDL	BDL
	Stem	BDL	BDL	BDL	BDL
	Root	3205 (168)	3202 (178)	3100 (129)	2853 (69)

Table 3. 3. Pigment concentrations (mg kg^{-1} FW) in jack pine treated with boron. Plants were grown in sand culture for four weeks. Values, n=6 represent mean (SE). There was no significant difference between treatments means at $\alpha = 0.05$ based on Duncan's Multiple Range Test (DMRT)

Pigment concentrations (mg kg^{-1} DW)	Treatment	
	Control	2.0 mM B
Chlorophyll *a*	380.87 (14.32)	400.27 (20.66)
Chlorophyll *b*	195 (13.97)	236 (28.56)
Total carotenoids	18.28 (1.02)	21.34 (1.30)

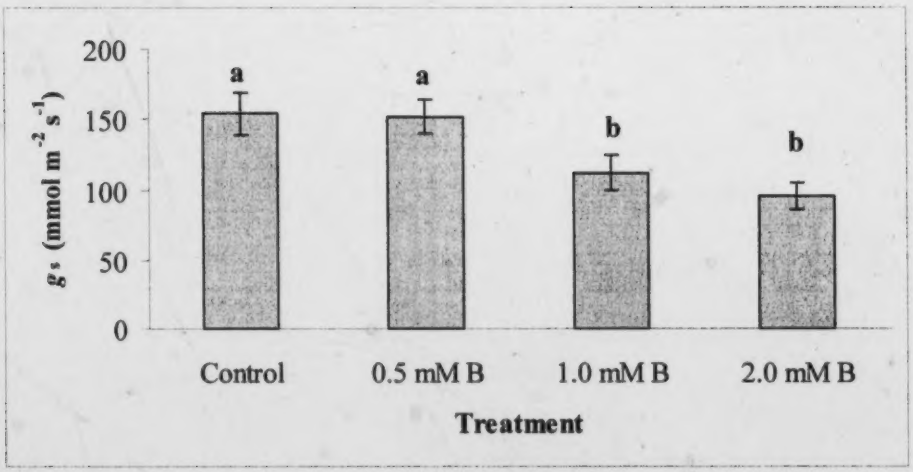

Figure 3. 1. Stomatal conductance (g_s) in jack pine seedlings treated for six weeks with boron. Bars represent means (n=6) ± SE. Bars with different letters indicate significant differences between treatments at $P \leq 0.05$ as determined by the Duncan's Multiple Range Test (DMRT).

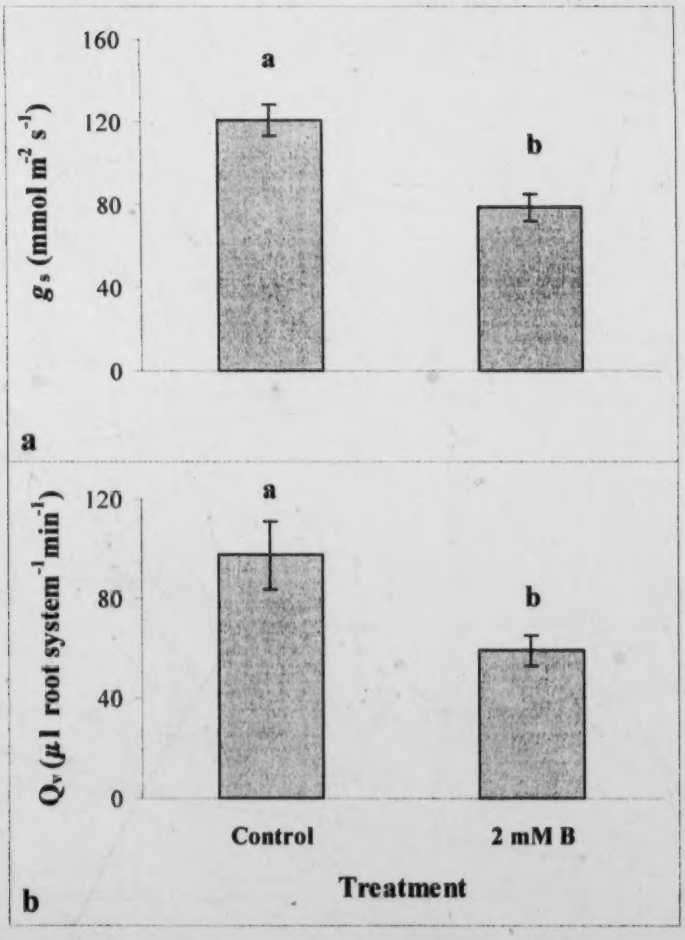

Figure 3. 2. a) Stomatal conductance (g_s) and b) root water flow (Q_v) in jack pine seedlings treated for four weeks with boron. Bars represent means (n=6) ± SE. Bars with different letters indicate significant differences between treatments at $P \leq 0.05$ as determined by the Duncan's Multiple Range Test. (DMRT).

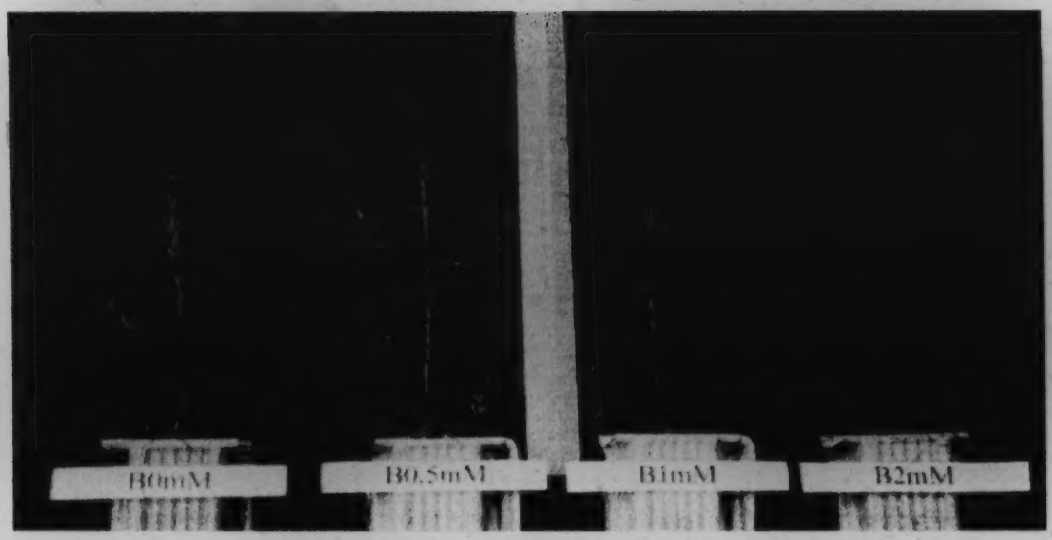

Figure 3. 3. Symptoms of B toxicity in jack pine seedlings treated for six weeks. Chlorosis and necrosis appeared first on the needle tips and margins and progressively developed towards the bases of the needles.

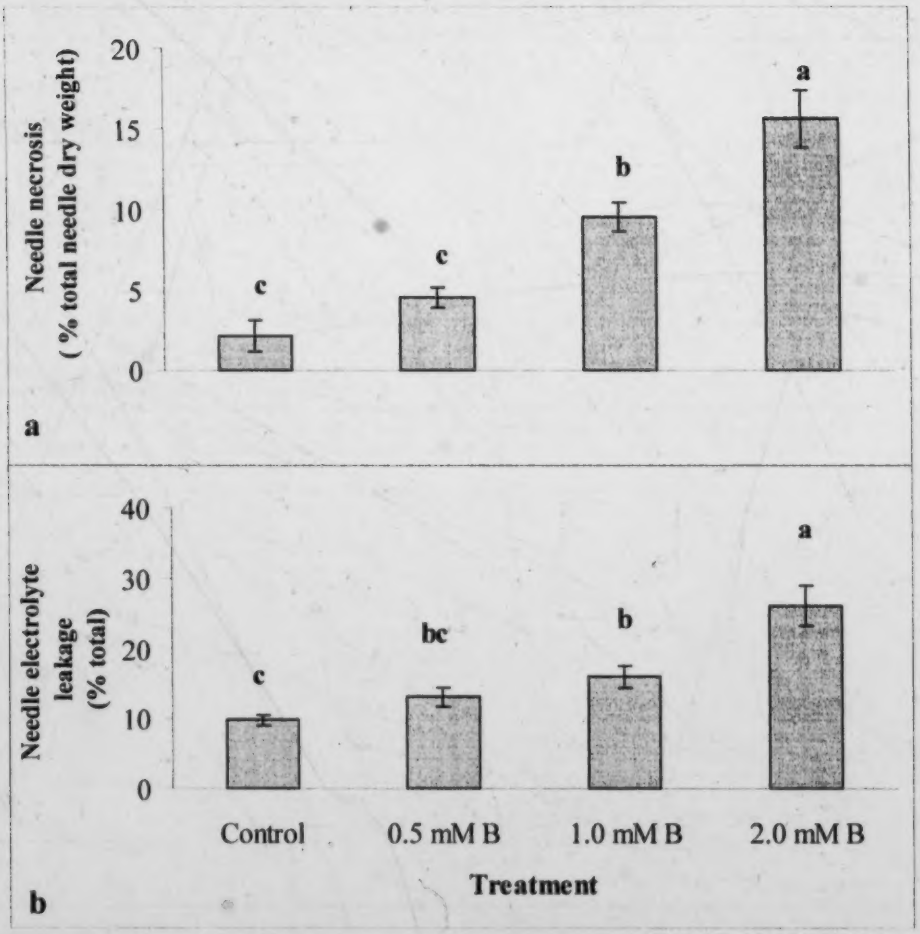

Figure 3. 4. a) Needle necrosis and b) needle electrolyte leakage in jack pine seedlings treated for six weeks with boron. Bars represent means (n=6) ± SE. Bars with different letters indicate significant differences between treatments at $P \leq 0.05$ as determined by the Duncan's Multiple Range Test (DMRT).

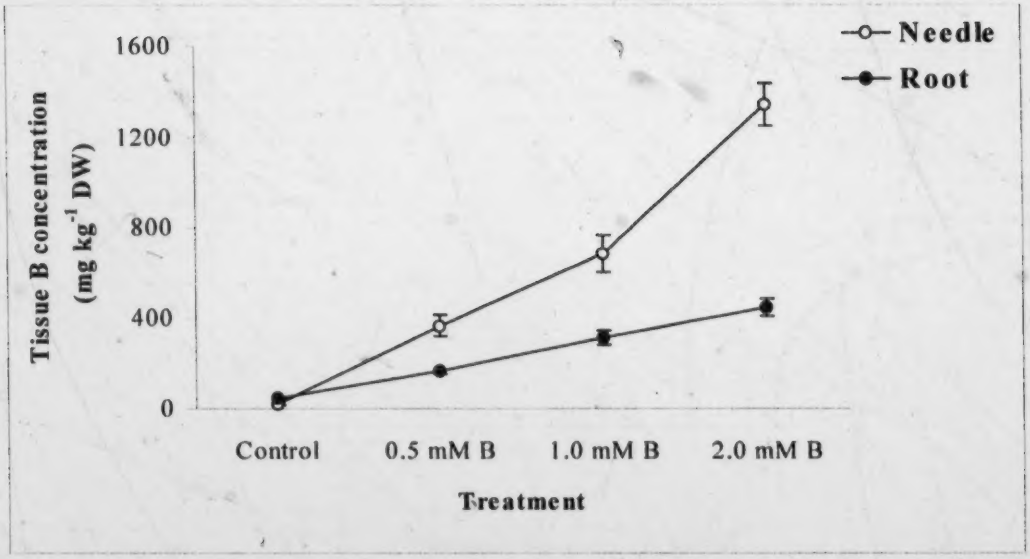

Figure 3. 5. Tissue boron concentrations (mg kg^{-1} DW) of jack pine seedlings treated for six weeks. Each data point represents mean (n=6) ± SE.

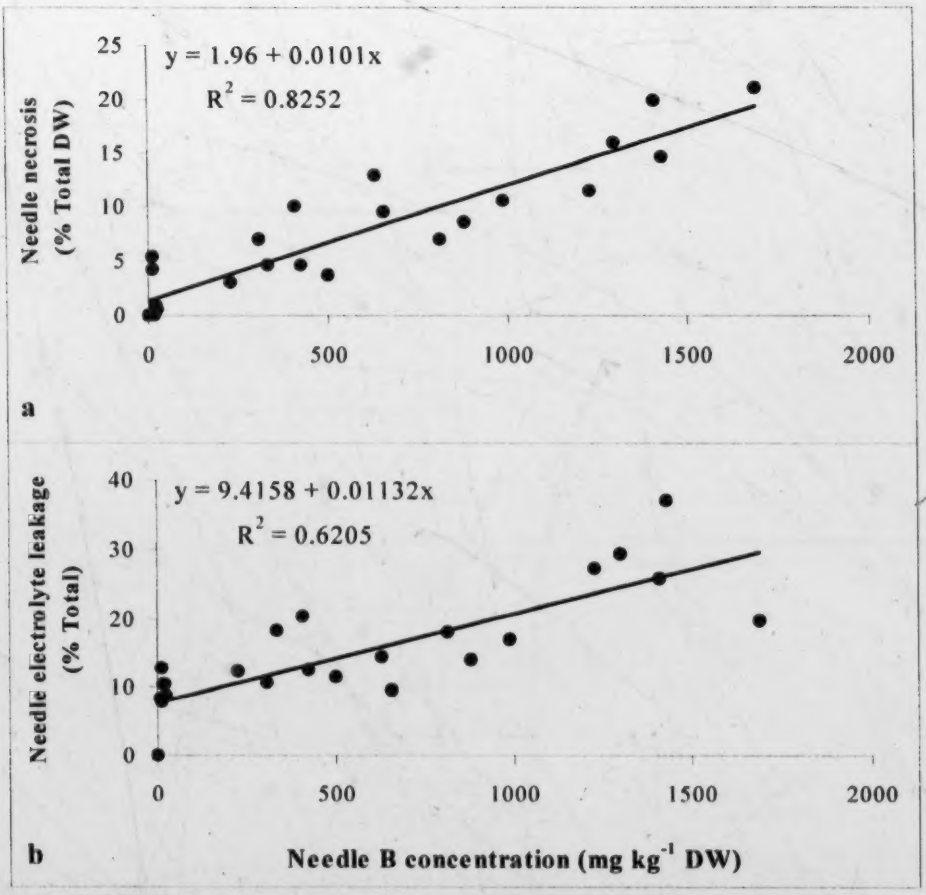

Figure 3. 6. Relationship between needle B concentrations and a) needle necrosis and b) needle electrolyte leakage in jack pine treated for six weeks. Each data point represents individual measurement of a single plant.

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3.6. Appendices

Table A.3. 1. ANOVA for survival, growth, stomatal conductance, needle necrosis and needle electrolyte leakage of jack pine seedlings treated with boron (0, 0.5, 1 and 2 mM) for six weeks, with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Percent survival	Boron	3	1.00	0.4547
	Rep	2	3.00	0.1250
	Error	6		
New shoot length	Boron	3	0.73	0.5478
	Rep	2	1.22	0.3177
	Error	15		
Number of new roots	Boron	3	1.17	0.3474
	Rep	2	1.23	0.3163
	Error	15		
Shoot fresh weight	Boron	3	0.13	0.9387
	Rep	2	1.82	0.1959
	Error	15		
Root fresh weight	Boron	3	1.16	0.3587
	Rep	2	3.56	0.0543
	Error	15		
Stomatal conductance (g_s)	Boron	3	5.44	0.0083
	Rep	2	0.33	0.7235
	Error	15		
Needle necrosis	Boron	3	22.19	<0.0001
	Rep	2	0.56	0.5826
	Error	15		
Needle electrolyte leakage	Boron	3	20.01	<0.0001
	Rep	2	4.78	0.0226
	Error	15		

Table A.3. 2. ANOVA for stomatal conductance and root hydraulic conductance of jack pine seedlings treated with boron for four weeks.

Parameter	SV	df	F	Pr>F
Stomatal conductance (g_s)	Boron	1	17.45	0.0019
	Error	10		
Root water flow (Q_v)	Boron	1	6.72	0.0269
	Error	10		
Chlorophyll *a*	Boron	1	2.58	0.0980
	Error	10		
Chlorophyll *b*	Boron	1	1.73	0.2175
	Error	10		
Total carotenoids	Boron	1	3.27	0.1005
	Error	10		

Table A.3. 3. ANOVA for tissue elemental composition of jack pine seedlings treated with boron (0, 0.5, 1 and 2 mM) for six weeks, with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Needle B	Boron	3	69.94	<0.0001
	Rep	2	0.44	0.6545
	Error	15		
Stem B[a]	Boron			
	Rep			
	Error			
Root B	Boron	3	51.65	<0.0001
	Rep	2	0.97	0.3994
	Error	15		
\Needle Ca	Boron	3	0.91	0.4573
	Rep	2	2.27	0.1376
	Error	15		
Stem Ca	Boron	3	1.30	0.3101
	Rep	2	1.01	0.3870
	Error	15		
Root Ca	Boron	3	1.97	0.1623
	Rep	2	0.41	0.6709
	Error	15		
Needle K	Boron	3	0.47	0.7091
	Rep	2	0.77	0.4804
	Error	15		
Stem K	Boron	3	0.64	0.5996
	Rep	2	0.23	0.7951
	Error	15		
Root K	Boron	3	0.86	0.4821
	Rep	2	0.71	0.5096
	Error	15		
Needle Mg	Boron	3	0.19	0.9021
	Rep	2	1.28	0.3055
	Error	15		
Stem Mg	Boron	3	1.25	0.3268
	Rep	2	3.17	0.0712
	Error	15		
Root Mg	Boron	3	1.65	0.2199
	Rep	2	1.43	0.2705
	Error	15		
Needle P	Boron	3	0.64	0.6010
	Rep	2	0.28	0.7585
	Error	15		
Stem P	Boron	3	0.90	0.4718
	Rep	2	1.38	0.2877
	Error	15		
Root P	Boron	3	4.37	0.2120
	Rep	2	1.70	0.2163
	Error	15		
Needle S[a]	Boron			
	Rep			
	Error			
Stem S[a]	Boron			
	Rep			
	Error			
Root S	Boron	3	1.48	0.2637
	Rep	2	1.53	0.2502
	Error	15		

[a] Not tested due to Below Detection Levels

Table A.3. 4. ANOVA for the regression equation of needle necrosis and needle B concentration.

SV	df	F	Pr>F
Regression	1	89.71	<0.0001
Error	19		

Table A.3. 5. Regression estimates and statistics of the regression equation of necrosis and needle B concentration.

Variable	df	Parameter estimate	T for Ho: parameter =0	Prob>T
Intercept	1	1.960	2.23	0.0378
Needle B concentration	1	0.010	9.47	<0.0001

Table A.3. 6. ANOVA for the regression equation of needle electrolyte leakage and needle B concentration.

SV	df	F	Pr>F
Regression	1	35.54	<0.0001
Error	20		

Table A.3. 7. Regression estimates and statistics of the regression equation of needle electrolyte leakage and needle B concentration.

Variable	df	Parameter estimate	T for Ho: parameter =0	Prob>T
Intercept	1	9.417	6.15	<0.0001
Needle B concentration	1	0.011	5.94	<0.0001

CHAPTER FOUR*

NaCl and Na_2SO_4 alter responses of jack pine (*Pinus banksiana*) seedlings to boron

4.1. Introduction

Problems with boron toxicity have been reported in many dry land areas in the world (Mahalakshmi et al. 1995). Potential sources of high B concentrations in soils are irrigation water, surface mining, fly ash, and industries (Aucejo et al. 1997; Nable et al. 1997). Boron is frequently associated with other salts (El-Motaium et al. 1994), most commonly in areas with poor drainage or with shallow water tables that limit opportunity for leaching (Gupta et al. 1985). In the northern boreal forest of Canada, continuing and planned development of the oil sands reserves will result in large areas containing substrates or waters containing elevated levels of B, NaCl and Na_2SO_4. These areas will require reclamation, and successful re-vegetation options must be available for those environments with high ionic loadings (Renault et al. 1998). A number of studies quantified the effects and examined the mechanisms of salt and B toxicity in plants (Levitt 1980; Munns 1993; Nable et al. 1997). However, little emphasis has been placed on understanding how the presence of other salts affects plant responses to B. Since B is often present in saline soils, it is important to understand the interactions of B and salts and their effects on plants.

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Boron toxicity in plants is associated with chlorosis and necrosis, usually at the tips and margins of the older leaves (Nable et al. 1997). Boron is transported with the transpiration stream from roots to leaves (Raven, 1980) where it affects cell metabolism. At higher concentrations, B alters cell wall cross linkages and inhibits cell wall expansion (Loomis and Durst 1992). In addition, when salinity is elevated, problems for plant growth and development resulting from altered physiological processes can be expected (Shannon et al. 1994). Greenway and Munns (1980) identified water deficit and direct ion toxicity as main mechanisms of salt stress in plants. While there is an abundance of information concerning effects of NaCl, relatively little is known about the responses of plants to Na_2SO_4, although, there are indications that NaCl is more phytotoxic than Na_2SO_4 (Ashraf and Yousaf 1998; Rogers et al. 1998).

In earlier studies, toxicity symptoms in plants affected by oil sands tailings containing elevated levels of sodium, chloride and sulfate, could not be attributed solely to salt stress (Renault et al., 1998). Since in addition to NaCl and Na_2SO_4, oil sands tailings contain relatively high concentrations of B (2-4 mg l^{-1} tailings) (Renault et al. 1999), it is possible that B aggravates salt-induced injury in plants. However, there are reports that salinity may reduce severity of B toxicity by lowering shoot and stem B accumulation (El-Motaium et al. 1994; Grattan et al. 1997; Rozema et al. 1992).

The principal objective of the present study was to determine the combined effects of B and isomolar concentrations of NaCl and Na_2SO_4 on the growth, injury and ion composition of jack pine (*Pinus banksiana* Lamb.) seedlings. Jack pine was used due to its relative susceptibility to B and salinity and its dominant ecological role in the northeastern Alberta (Renault et al. 1998; Renault et al. 1999). The hypotheses that B

toxicity adds to NaCl and Na_2SO_4 effects on plant growth and that the combined effects of B and salt treatments are more severe compared with the individual treatment effects were tested.

4.2. Materials and methods

4.2.1. Plant material and treatments

Six-month-old dormant, container-grown jack pine seedlings were obtained from the Pine Ridge Forest Nursery, Smoky Lake, Alberta. Seedlings were re-planted into 200-ml Spencer-Lemaire root trainers filled with coarse washed sand and arranged in 4-l pots (6 seedlings per pot), which were placed in 10-l plastic containers and soaked in nutrient solution containing 80 mg l^{-1} N, 60 mg l^{-1} P, 104 mg l^{-1} K, 100 mg l^{-1} Ca, 60 mg l^{-1} Mg, 79 mg l^{-1} S, 3 mg l^{-1} Fe, 0.40 mg l^{-1} Mn, 0.25 mg l^{-1} B, 0.14 mg l^{-1} Zn, 0.50 mg l^{-1} Cu and 0.10 mg l^{-1} Mo. Seedlings were transferred into a growth room set to the following environmental conditions: day/night temperature, 22/18°C; relative humidity, 70%; 18-h photoperiod and photosynthetic flux density at the seedling level of approximately 300 μ mol m^{-2} s^{-1}. After one week, seedlings were randomly split into 6 groups and the containers were filled with 3 L of the following 6 treatment solutions: control (nutrient solution), 2 mM H_3BO_3, 60 mM NaCl, 2mM H_3BO_3 + 60 mM NaCl, 60 mM Na_2SO_4 and 2 mM H_3BO_3 + 60 mM Na_2SO_4. All treatments were prepared in nutrient solution. The study was conducted for four weeks and the design was randomly replicated three times with each container containing six seedlings per pot (Figure A.4.1).

Seedlings in pots were irrigated in containers with treatment solutions for 24 of every 48 hours. Seedlings were removed from the containers to avoid flooding stress. Solutions were replaced once a week and containers covered with lids when the seedlings were out of the solution culture to prevent growth of algae and evaporation. Seedlings remained out of the solution culture for a day. A 3-L volume of solution was maintained by replenishing with distilled water.

4.2.2. Plant survival and growth

Survival was monitored daily until the harvest date, four weeks after the commencement of treatments. At harvest, lengths of new shoots were measured and six seedlings were randomly harvested from each treatment to count the number of new roots ≥ 50 mm in length. Dry weights of shoots and roots were determined following freeze-drying for 72 h and shoot to root ratio was calculated.

4.2.3. Transpiration

After four weeks of treatment initiation, transpiration rates, which were measured on the upper 30-mm terminal shoots of the seedlings using a steady state poromoter LI-600 (LI-COR Inc., Nebraska, USA), were obtained from six randomly selected seedlings from each treatment. Measurements were conducted in the growth room between 3 to 4 hours after the onset of the photoperiod and 23 to 24 hours after irrigation. The relative

humidity inside the cuvette was set at 50%. Needle surface area was obtained following computer scanning (Sigma Scan 4.01, SPSS, Chicago, IL).

4.2.4. Assessment of visible toxicity symptoms and electrolyte leakage

Visible injury was monitored daily until the harvest date. After four weeks, needle necrosis was assessed in 8 randomly selected needles per seedling and the needles with severe necrosis were counted. Needles exhibiting severe necrosis were considered for measurements when the length of necrotic areas was greater than or equal to 45 mm.

For the electrolyte leakage test, six seedlings per treatment were randomly harvested and washed with deionized water three times, for five minutes each. The electrolyte leakage test is a method that detects the loss of electrolyte from semi-permeable cell membranes (Zwiazek and Shay, 1988) as described by Renault et al., (1998). Briefly, samples containing 0.45 g of needles were placed in tubes, each containing 10 ml of deionized water. After 1-h incubation, the solutions were replaced with another 20 ml of deonized water and incubated for 5 h at 50 RPM in an orbital shaker. Electrical conductivity of the solutions was measured with an electrical conductivity meter HI 8033 (Hanna Instruments Inc., Woonsocket, RI). Total electrolytes remaining in tissue were obtained by autoclaving the samples at 121 ^{o}C followed by freezing overnight at –85^{o}C. The samples were then thawed by placing them in the water bath at room temperature for 5 h. The electrolyte leakage was expressed as percentage of the total electrolyte content.

4.2.5. Elemental analysis of plant tissues

Freeze-dried shoots and roots were plunged into liquid nitrogen and finely ground using a mortar and pestle prior to elemental analysis. Separate samples of roots and shoots were used to determine the cations and anions present in both tissues. The preparation for analysis of Cl^- and SO_4^{2-} was based on a hot water extraction technique, where the plant tissue sample (0.05 g) is leached with 10 ml of hot (70-80 °C) water. The aqueous mixture was shaken for 20-30 minutes, and then centrifuged at 2000 RPM for 20 minutes. This extraction was repeated and the first and second extracts were combined and filtered with a 45-μm Millipore filter, and then analyzed using ion chromatography (Dionex-300 Series, Dionex Corp., Sunnyvale, CA). A series of extractions was done to verify the completeness and accuracy of extraction using this technique. Results showed that with two-staged extraction essentially all the readily leachable ions were obtained.

Boron and the remaining analyzed tissue elements/ions concentrations were determined using the ICP-OES method (Vista-RL CCD Simultaneous ICP-OES, Varian Inc., Victoria, Australia) after strong acid (5 % HNO_3) digestion as previously described (Renault et al., 1999).

4.2.6. Statistical analysis

Statistical analysis was performed using SAS general linear model (SAS Institute Inc., Cary, NC 1996) and treatment means were compared using Duncan's multiple range test (DMRT) at the $P \leq 0.05$ level. The following model was used:

$$Y_{ij} = \mu + \rho_i + \tau_j + \varepsilon_{ij}$$

where:
Y_{ij} = response variable
μ = overall mean
ρ_i = replication (random factor), i = 1…3;
τ_j = treatment effect (fixed factor), j =1…6; and
ε_{ij} = random error

4.3. Results

4.3.1. Plant survival and growth

When applied separately, treatments with 60 mM NaCl and 60 mM Na_2SO_4 significantly reduced survival of jack pine seedlings relative to control seedlings following 4 weeks of exposure (Figure 4. 1A). When B was added to either salt treatment, seedling survival decreased significantly ($P \leq 0.05$) (Figure 4. 1A).

Seedlings in all treatments containing NaCl or Na_2SO_4 showed a significant reduction in the length of new shoots (Figure 4. 1B) and in the number of new roots (Figure 4. 1C) compared with control plants. The number of new roots ≥ 5 mm in seedlings was significantly reduced from 10 in control plants to 0.83 and 1.5 in Na_2SO_4 and NaCl-treated plants, respectively (Figure 4. 1C). No new roots were produced in B +

NaCl and B + Na_2SO_4 treatments. Shoot to root ratio significantly decreased only in Na_2SO_4 and Na_2SO_4 + B treatments (Figure 4. 1D).

Transpiration rates were significantly lower values in plants treated with B + salt. Transpiration rate was approximately 2.5 mmol H_2O m^{-2} s^{-1} in B + NaCl and B + Na_2SO_4-treated plants compared with 5.2 mmol H_2O m^{-2} s^{-1} in control plants (Figure 4. 2A).

4.3.2. Visible injury and electrolyte leakage

Visible injury was only observed in needles. Extensive needle chlorosis was first observed in B + NaCl, two weeks after the treatment and it gradually changed into necrosis. At the end of the experiment, B-treated plants demonstrated some needle tip necrosis ranging from 5-24 mm in length. In 2mM B + 60 mM NaCl, seedlings exhibited severe needle necrosis (Figure 4. 2B).

Needle electrolyte leakage was not significantly altered in plants treated with 2 mM B (Figure 4. 2C). However, 60 mM NaCl and 60 mM NaCl + 2 mM B increased electrolyte leakage by more than 80% compared with control plants (Figure 4. 2C). Treatment with 2 mM B + 60 mM Na_2SO_4 treatment also resulted in an increase in needle electrolyte leakage by 78% compared with control plants (Figure 4. 2C).

4.3.3. Elemental composition in shoots and roots

Ion concentrations measured in the plant tissues have been reported as mg of element per g of dry weight (DW) in the freeze-dried tissue.

Boron concentration in the shoot tissue of seedlings treated with 2 mM B was 24% higher than that in the roots (Figure 4. 3A). Shoots of B-treated plants contained 2.4 mg B g^{-1} DW. A large reduction (>75%) in shoot B concentration was observed in 2 mM B + 60 mM NaCl and 2 mM B + 60 mM Na_2SO_4 treatments compared to the 2 mM B treatment (Figure 4. 3A). At the same time, the amount of B measured in the roots showed an opposite trend, with a 10-20% increase in B + salts treatment versus B alone. As a result, the relative concentration of B in various parts of the plant was reversed. This meant that in the 2 mM B treatment, the relative distribution between the shoots and roots was about 55:45, while in the treatments that also included NaCl and Na_2SO_4, the ratio was about 25:75 shoots to roots (Figure 4. 3A).

Seedlings treated with 60 mM Na_2SO_4 had significantly higher root Na concentration than those treated with 60 mM NaCl (Figure 4. 3B). However, shoot Na concentrations of Na_2SO_4-treated plants were lower than the shoot Na concentration of the plants treated with NaCl. Boron present in both salt treatments altered the distribution of tissue Na by reducing Na concentration in the shoots. This effect of boron was not observed for Cl^- (Figure 4. 3C). The root levels of SO_4 increased in Na_2SO_4-treated plants in the presence of B (Figure 4. 3D).

In both shoots and roots, Ca concentrations were reduced by the salt treatments and little changed in plants treated with B without salt (Figure 4. 4A). A significant

reduction of Mg concentration was observed only in the 60 mM Na_2SO_4 treatment in roots (Figure 4. 4B). Similarly, a drastic reduction of K concentration was observed only in the roots of salt-treated plants and did not change by the B treatment alone (Figure 4. 4C).

4.4. Discussion

With the exception of root growth (Figure 4. 1B), 2 mM B treatment for four weeks had little effect on the measured growth parameters in jack pine seedlings. However, when added to 60 mM NaCl and 60 mM Na_2SO_4 treatments, B decreased seedling survival, increased needle injury and altered tissue elemental composition in seedlings.

I observed some needle tip necrosis in seedlings treated with 2 mM B for four weeks. However, other than reduced root growth, I did not observe B toxicity symptoms in the roots. This absence of root injury, even at high B concentrations, was also reported for other plants (Nable 1988). Since in the present study, B root concentrations were only slightly lower than those in the shoots, the absence of root injury in B-treated plants suggests that roots may respond differently to B than shoots.

My data indicate that both NaCl and Na_2SO_4 treatments reduced B transfer from roots to shoots (Figure 4. 3A). This likely occurred as a result of reduction in transpiration, since there was a significant negative correlation between shoot B concentration and transpiration rates in B-treated plants ($P \leq 0.05$, $r = -0.793$). A decline in transpiration is a common response of jack pine and other boreal plants to salinity (Renault et al. 1999), and occurs either due to partial stomatal closure or reduction in water uptake (Hagemeyer 1997). The decrease in B concentration of salt-treated plants is

likely responsible for reduced B toxicity symptoms reported in other studies (El-Motaium et al. 1994; Marcar et al. 1999).

In my study, transpiration rates were further reduced by Na_2SO_4 compared with NaCl treatments, likely an osmotic effect due to lower osmotic potential of 60 mM Na_2SO_4 compared with 60 mM NaCl (Hagemeyer 1997). Although it may be difficult to distinguish between the osmotic effect and the specific ion effect using isomolar salt treatments, other studies (Warne et al. 1990; Redfield 2000) also demonstrated that, as opposed to NaCl, the effects of Na_2SO_4 on plants could be largely explained by osmotic stress.

In the present study, B + NaCl resulted in greater needle necrosis (Figure 4. 2B), electrolyte leakage (Figure 4. 2C) and reduced shoot length (Figure 4. 1B), compared with B + Na_2SO_4-treated seedlings. Redfield (2000) explained early effects of NaCl on conifer seedlings as a result of ionic toxicity. One mechanism for the control of salt excess in a metabolically active leaf is preferential distribution of ions in various plant parts (Shannon et al. 1994). The low injury and lesser reduction of growth in Na_2SO_4 treatment, compared with NaCl, could be partly due to the ability of jack pine to preferentially accumulate SO_4^{2-}, Na and B in roots rather than shoots. It is also possible that SO_4^{2-} could help alleviate Na toxicity (Harborne 1977). However, a potential protective role of SO_4^{2-} in salt stress remains unclear.

Using isomolar concentrations of salts allowed us to assess relative sensitivities of jack pine to Cl^- and Na. The concentration of Na in 60 mM Na_2SO_4 treatment solution was twice as high as in 60 mM NaCl solution. However, the jack pine seedlings treated with 60 mM Na_2SO_4 exhibited less injury compared with seedlings exposed to 60 mM

NaCl, suggesting that there was a factor other than Na contributing to salt toxicity. A similar pattern was also observed in salt + B treatments. The results suggest that Cl^- has a significant contribution to salt injury in jack pine.

Jack pine shoots accumulated more Cl^- than Na (Figure 4. 3B, Figure 4. 3C). The inability of jack pine seedlings to restrict Cl^- shoot uptake may be linked to the mechanisms associated with NaCl toxicity. It has been found that an accumulation of Cl^- in the leaf tissues of *Citrus* plants caused a large reduction in photosynthesis and stomatal conductance, while these parameters were less affected by Na (Bañuls and Primo-Millo 1992). The ability to maintain low foliar Cl^- concentration was also likely a major factor contributing to NaCl tolerance in *Trifolium alexandrium* L. (Winter and Lauchli 1982).

In my study, the enhancement of electrolyte leakage and needle necrosis in NaCl-treated plants by B occurred without corresponding increase in B, Na or Cl^- concentrations. In fact, shoot B levels in B + NaCl-treated plants were lower than these measured in seedlings treated with B without NaCl. These results suggest possible synergistic effects between B and NaCl on cell membrane integrity. The electrolyte leakage method is used as an indicator of cell membrane injury (Zwiazek and Blake 1991) since the apoplastic electrolytes are removed by incubating the tissue in deionized water.

Salts cause nutritional imbalance by competitive interactions between ions and membrane selectivity (Cramer et al. 1985). Effects of salinity on nutrient availability and distribution of Ca in treated plants is particularly interesting because Ca plays an important role in maintaining cell membrane integrity, controlling membrane permeability and improving resistance of plants to salt stress (Azaizeh et al. 1992;

Marschner 1995; Carvajal et al. 2000; Kent and Lauchli 1985). The present results are consistent with the earlier findings (Renault et al. 1999) that showed reductions in Ca^{2+} and Mg^{2+} concentrations of Na_2SO_4-treated plants. High concentration of Ca in NaCl–treated plants probably help in counterbalancing the accumulated Cl^-. Recent studies also showed that Ca alleviates inhibition of water channel activity in NaCl-treated plants (Carvajal et al. 2000). Other studies also confirmed the ameliorating effect of Ca by increasing B retention in root tips thereby reducing transport to the stem and leaves (El-Motaium 1994). The complete loss of root K (Figure 4. 4C) may result from competitive inhibition of K uptake at high Na concentration (Smart and Barko 1980) or inhibition resulting from loss of cell membrane integrity (Greenway and Munns 1980) which leads to K efflux into the growth medium (Cramer et al. 1985).

In conclusion, my results have shown that the 2 mM B treatment for 4 weeks reduced new root growth, but had little effect on other growth variables, transpiration rates, needle integrity and tissue elemental composition. However, when added to 60 mM NaCl and Na_2SO_4 treatments, B aggravated growth reduction and injury induced by these salts. Based on the electrolyte leakage and necrosis, Cl^- appeared to be the major factor contributing to seedling injury. I suggest that Cl^- contributed to Na and B toxicity to jack pine seedlings by affecting cell membrane integrity.

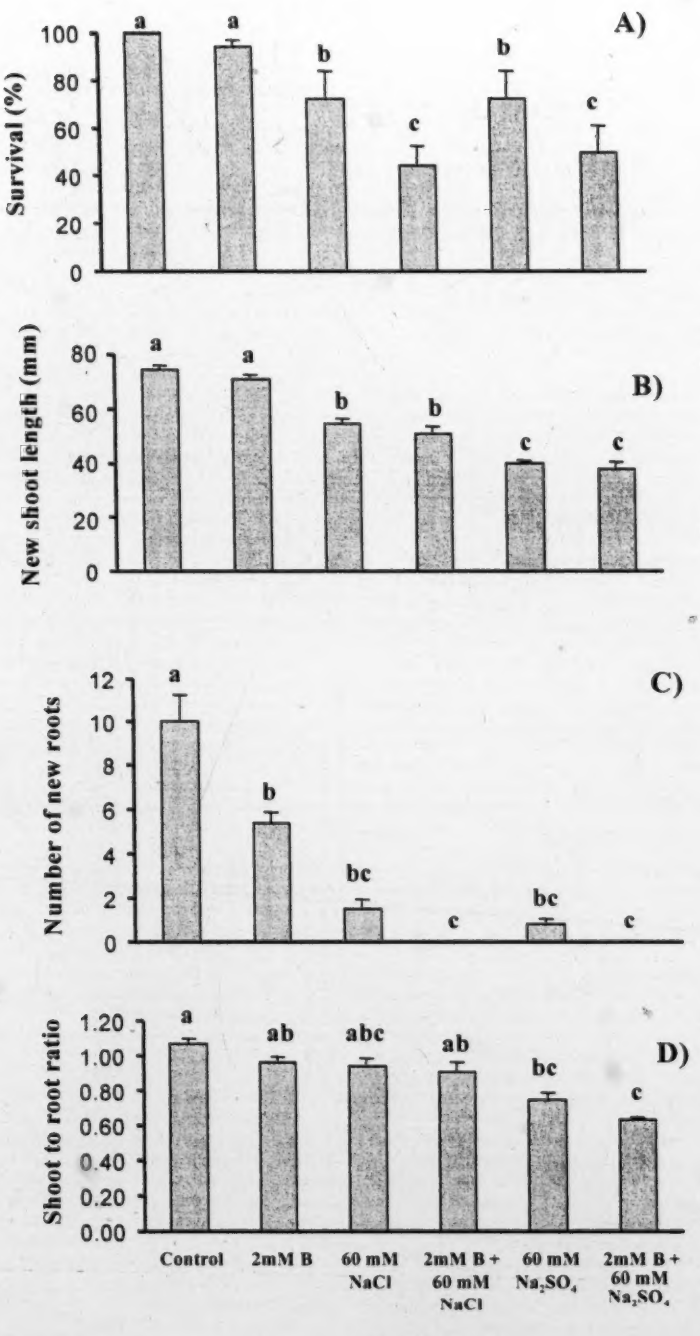

Figure 4. 1. Effects of B, NaCl, and Na_2SO_4 on A) survival, B) new shoot length (mm), C) number of new roots (≥ 5 mm); and D) shoot to root ratio of six-month-old jack pine seedlings. Seedlings were grown in sand culture and treated for four weeks. Bars are the means and SEM (n=6). Bars with different letters are significantly different from each other at α = 0.05 based on the Duncan's multiple range test (DMRT).

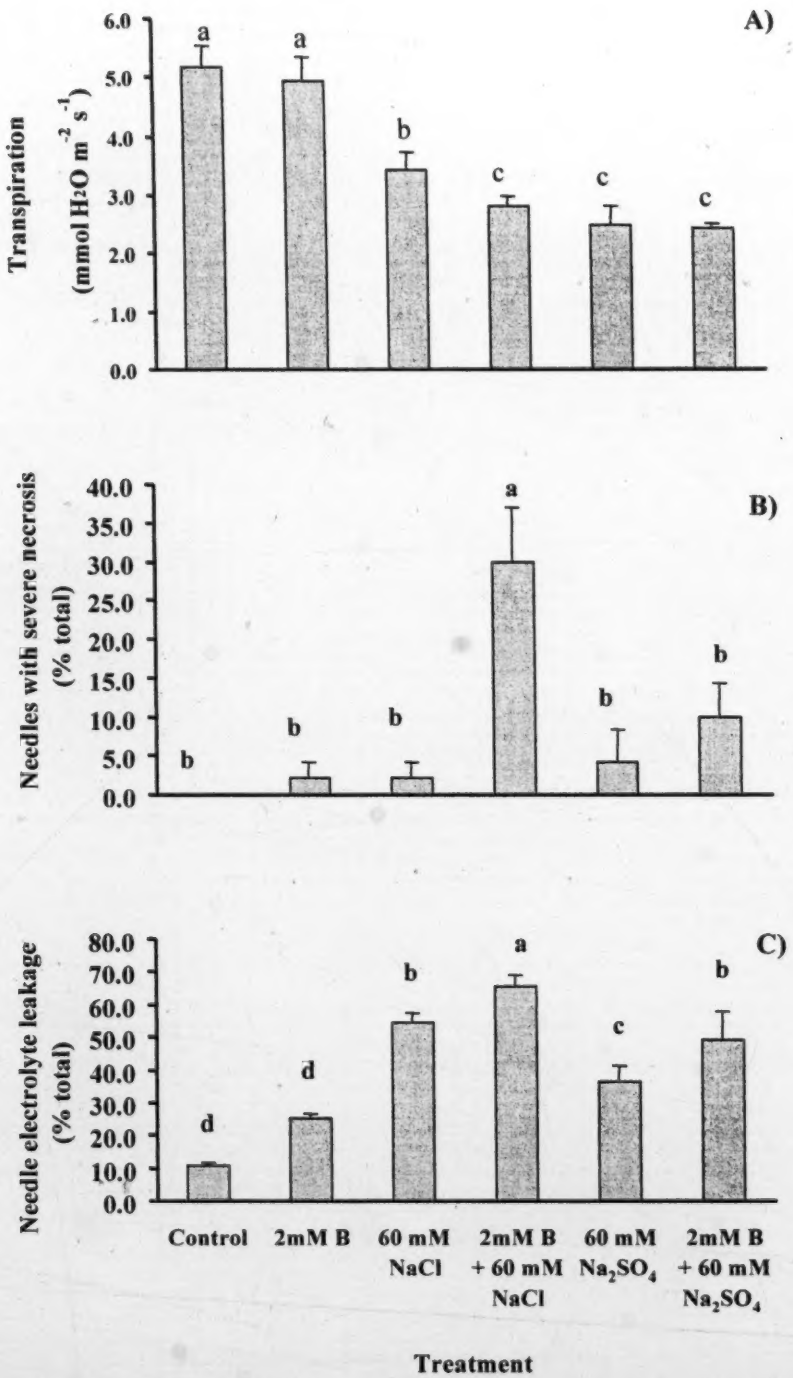

Figure 4. 2. Effects of B, NaCl Effects of B, NaCl, and Na_2SO_4 on A) transpiration, B) number of needles with severe necrosis; and C) electrolyte leakage rate of six-month-old jack pine seedlings. Seedlings were grown in sand culture and treated for four weeks. Bars are the means and SEM (n=6). Bars with different letters are significantly different from each other at $\alpha = 0.05$ based on the Duncan's multiple range test (DMRT).

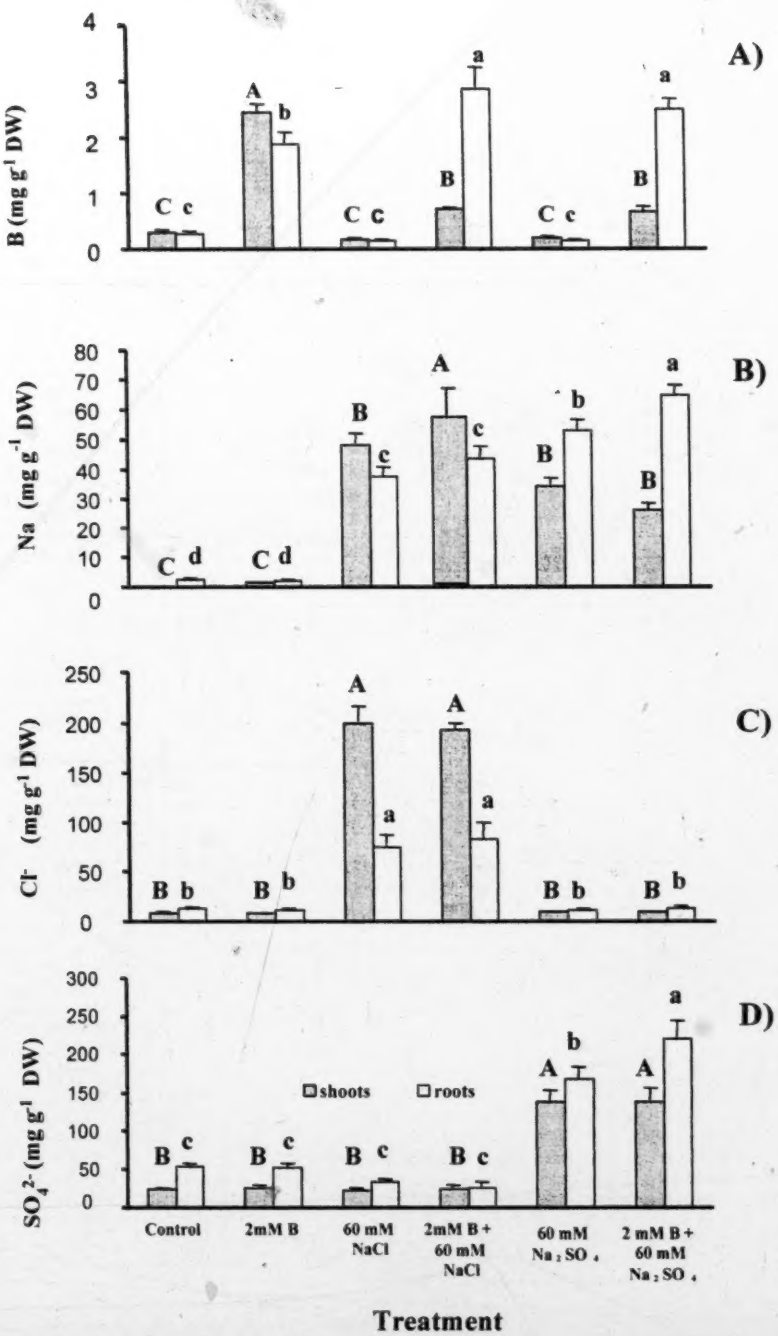

Figure 4. 3. Effects of B, NaCl, and Na_2SO_4 on shoot and root concentrations of A) boron, B) sodium, C) chloride; and D) sulfate concentrations in six-month-old jack pine seedlings. Seedlings were grown in sand culture and treated for four weeks. Bars are the means and SEM (n=6). * Bars with different letters are significantly different from each other at α = 0.05 based on the Duncan's multiple range test (DMRT). The uppercase letters are for treatment comparisons for shoots, and lowercase letters are for roots.

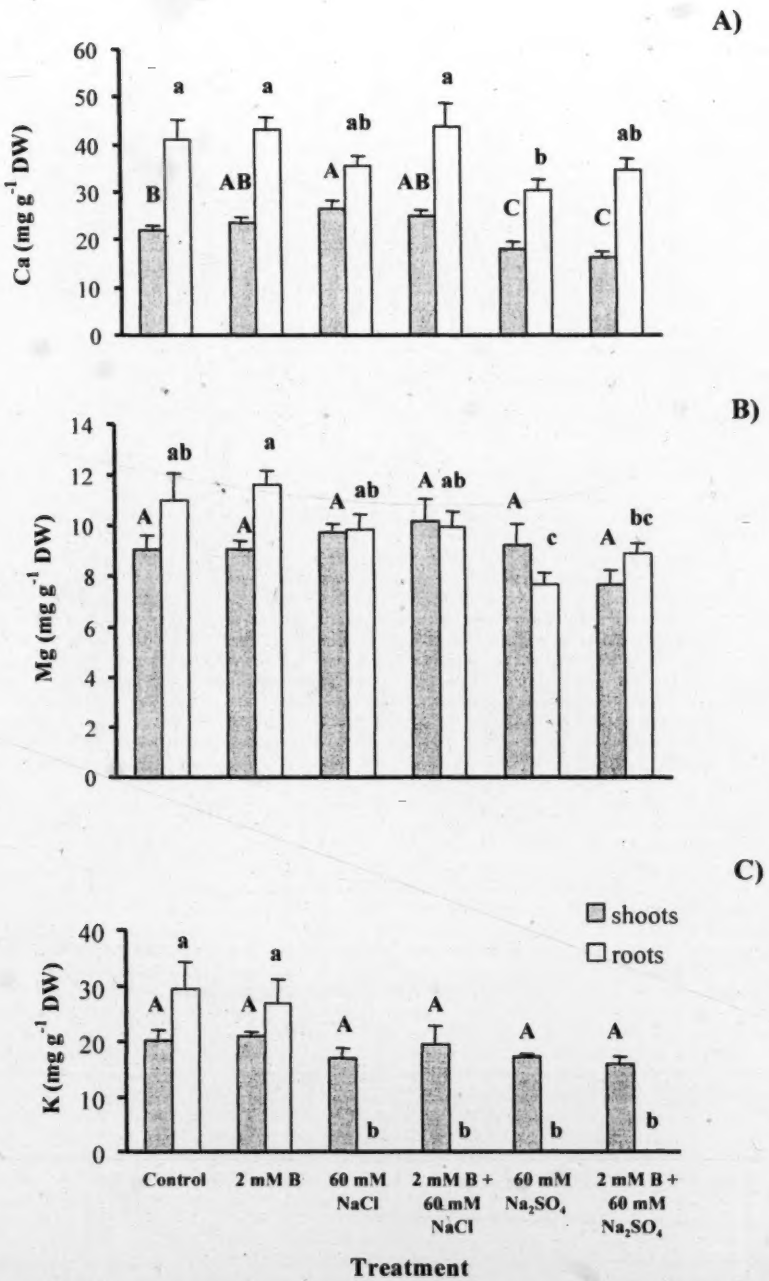

Figure 4. 4. Effects of B, NaCl, and Na_2SO_4 on shoot and root concentrations of A) calcium B) magnesium and C) potassium concentrations in six-month-old jack pine seedlings. Seedlings were grown in sand culture and treated for four weeks. Bars are the means and SEM (n=6). * Bars with different letters are significantly different from each other at $\alpha = 0.05$ based on the Duncan's multiple range test (DMRT). The uppercase letters are for treatment comparisons for shoots, and lowercase letters are for roots.

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4.6 Appendices

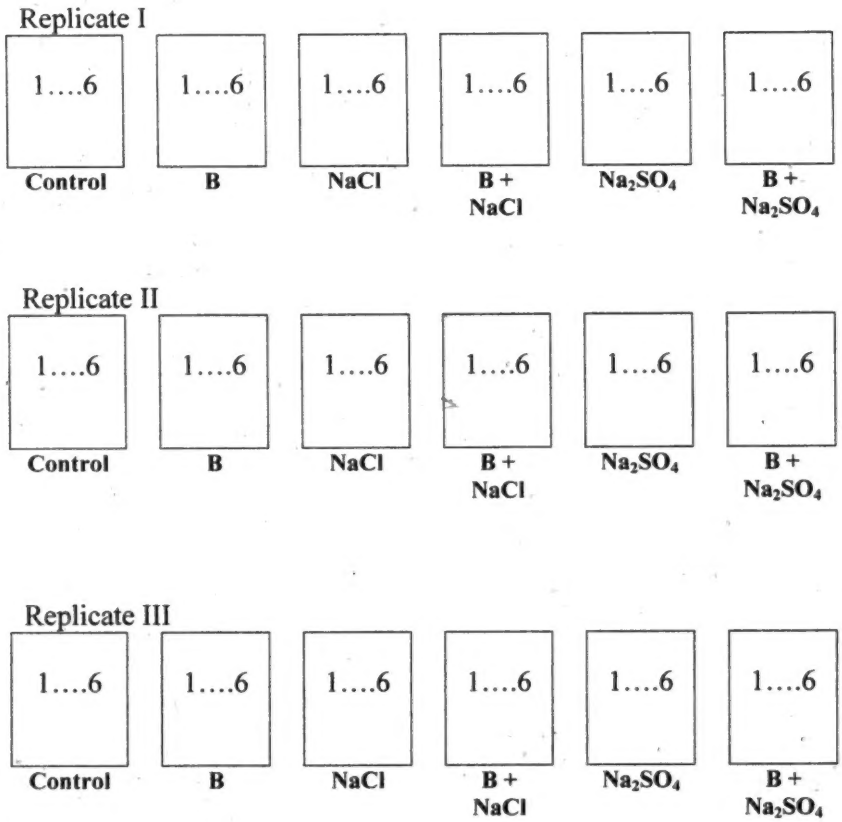

Figure A.4.1 Experimental layout of the study. Number (n=6) represents the number of seedlings that were placed in each treatment container, which was replicated three times.

Table A.4. 1 ANOVA for survival, growth, transpiration, needle necrosis and needle electrolyte leakage of jack pine seedlings treated with boron and salts, with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Percent survival	Treatment	5	3.35	0.0429
	Rep	2	4.39	0.0429
	Error	10		
New shoot length	Treatment	5	12.66	<0.0001
	Rep	2	7.62	0.0013
	Error	26		
Number of new roots	Treatment	5	7.39	0.0002
	Rep	2	1.49	0.2441
	Error	26		
Shoot to root ratio	Treatment	5	2.79	0.0382
	Rep	2	2.33	0.1176
	Error	26		
Transpiration	Treatment	5	3.90	0.0094
	Rep	2	7.13	0.0035
	Error	26		
Number of needles with severe necrosis	Treatment	5	23.67	<0.0001
	Rep	2	0.33	0.7204
	Error	26		
Needle electrolyte leakage	Treatment	5	3.73	0.0111
	Rep	2	2.44	0.1065
	Error	26		

Table A.4. 2 ANOVA for tissue elemental composition of jack pine seedlings treated with boron and salts, with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr<F
Shoot B	Treatment	5	105.10	0.0001
	Rep	2	0.45	0.6426
	Error	26		
Root B	Treatment	5	44.86	0.0001
	Rep	2	0.73	0.4906
	Error	26		
Shoot Na	Treatment	5	34.83	0.0001
	Rep	2	0.32	0.7280
	Error	26		
Root Na	Treatment	5	87.61	0.0001
	Rep	2	4.19	0.0264
	Error	26		
Shoot Cl	Treatment	5	154.38	0.0001
	Rep	2	0.89	0.4225
	Error	26		
Root Cl	Treatment	5	24.47	0.0001
	Rep	2	4.03	0.0298
	Error	26		
Root SO_4	Treatment	5	37.88	0.0001
	Rep	2	0.06	0.9392
	Error	26		
Shoot SO_4	Treatment	5	36.03	0.0001
	Rep	2	1.12	0.3406
	Error	26		
Shoot Ca	Treatment	5	8.77	0.0001
	Rep	2	0.35	0.7100
	Error	26		
Root Ca	Treatment	5	2.76	0.0396
	Rep	2	0.37	0.6928
	Error	26		
Shoot Mg	Treatment	5	1.79	0.1491
	Rep	2	0.19	0.8259
	Error	26		
Root Mg	Treatment	5	4.40	0.0049
	Rep	2	0.11	0.8931
	Error	26		
Shoot K	Treatment	5	1.65	0.1918
	Rep	2	3.47	0.0498
	Error	26		
Root K	Treatment	5	3.54	0.0263
	Rep	2	0.69	0.5259
	Error			

CHAPTER FIVE*

Hypoxia affects root sodium and chloride concentrations and alters water conductance in salt-treated jack pine (*Pinus banksiana*) seedlings

5.1. Introduction

Many stress resistance mechanisms in plants rely on the energy supplied by respiration. Therefore, when environmental stresses occur under oxygen deficient conditions, plants may not be able to use some of these mechanisms to cope with the stress (Jackson and Drew 1984; Drew and Läuchli 1985; Drew et al. 1988). Under saline conditions, both osmotic effects and ion toxicity contribute to injury and growth reduction in plants (Munns and Termaat 1986). Some plants tolerate saline environments through osmotic adjustment, restriction of salt uptake, or restriction of salt translocation to the shoot (Waisel 1991). These tolerance mechanisms are likely affected when roots experience oxygen deficiency.

Salt and flooding stresses upset water relations and result in the development of mineral deficiencies in sensitive plants (Zhang and Tyerman 1991; Kamaluddin and Zwiazek 2001). Both NaCl (Azaizeh et al. 1992; Carvajal et al. 1999) and hypoxia (Zhang and Tyerman 1999; Kamaluddin and Zwiazek 2002) inhibit root water flow, likely through their effects on the function of aquaporins (Kamaluddin and Zwiazek 2002). Therefore, when present concurrently with salt, hypoxia may exacerbate the

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problem of root water flow inhibition in plants caused by NaCl (Carvajal et al. 1999; 2000).

Both water deficits and ion accumulation are among the factors that cause reductions in plant growth under saline conditions and which may also be affected by hypoxia (Barrett-Lennard et al. 1999). The development of root oxygen deficiency conditions may exacerbate ion toxicity by affecting the ability of plants to regulate ion transport. Therefore, it is important to understand how plants that may be exposed to hypoxic conditions will respond to the presence of salts in the root zone.

Salinity and hypoxic conditions may affect revegetation of industrial and mining areas. In northeastern Alberta, Canada, elevated levels of salts are present in areas reclaimed after open-pit oil sands mining (Renault et al. 1998; 1999). Ionic loads in the high salinity tailings exert injurious effects on plants and thus hinder successful revegetation (Renault et al. 1998). Hypoxic conditions are likely to be present in many oil sands reclamation sites due to high water levels and as a consequence of methane diffusion from tailings deposit present below the root zone (Fedorak et al. 2000). Methane diffusion is expected to displace oxygen from the soil and produce temporary hypoxic conditions in the root zone (Fedorak et al. 2000). Therefore, plants in the reclaimed areas will have to cope with salinity under hypoxic conditions.

The objective of the present study was to examine the responses of jack pine (*Pinus banksiana* Lamb.) to the combined effects of NaCl and hypoxia. I tested the hypothesis that hypoxia aggravates plant responses to NaCl by inhibiting plant water transport and decreasing the ability of roots to sequester salt. I selected jack pine due to its importance for the revegetation of oil sands mining areas (Renault et al. 1998; 1999).

5.2. Materials and methods

5.2.1. Plant material and growth conditions

Seeds of jack pine (*Pinus banksiana* Lamb.) were collected from trees approximately 60 km north of Ft. McMurray, Alberta (57°05.95N 111°38.90W). The seeds were germinated and seedlings grown for six months in Spencer-Lemaire root trainers (350 ml volume, Steuwé and Sons, Inc., Corvallis, OR, USA) filled with a mixture of peat moss and sand (2:1 by volume). The seedlings were grown in a growth room that was set to the following environmental conditions: day/night temperature, 22/18°C; relative humidity, 70%; 18-h photoperiod with photosynthetic photon flux density of approximately 300 μmol m^{-2} s^{-1} at the seedling level. After six months, roots were rinsed in water and the seedlings were transferred to 10-l containers filled with aerated mineral nutrient solution containing 80 mg l^{-1} N, 60 mg l^{-1} P, 104 mg l^{-1} K, 100 mg l^{-1} Ca, 60 mg l^{-1} Mg, 79 mg l^{-1} S, 3 mg l^{-1} Fe, 0.40 mg l^{-1} Mn, 0.25 mg l^{-1} B, 0.14 mg l^{-1} Zn, 0.50 mg l^{-1} Cu and 0.10 mg l^{-1} Mo as previously described (Apostol et al. 2002). The seedlings were grown in solution culture for two weeks before the commencement of treatments.

5.2.2.Treatments

The experiment had a randomized block design with two NaCl concentrations (0 and 45 mM) and two aeration levels (dissolved O_2 concentrations of approximately 8 mg l^{-1} and 2 mg l^{-1} for well-aerated and hypoxic treatments, respectively). Salt stress was imposed by adding NaCl to the nutrient solution. To prevent osmotic shock, initially, 15 mM NaCl was supplied for one day and an additional 15 mM was added each day for two more days until a final concentration of 45 mM was achieved. I used the concentration of NaCl which is expected to be temporarily present in some oil sand reclamation areas. Aeration was provided by aquarium pumps (Optima model 807, Rolf C. Hagen Inc., Montreal, Canada). For well-aerated solutions, air valves were maintained fully open resulting in dissolved O_2 concentrations of approximately 8 mg l^{-1} while for hypoxic solutions, air valves were partly closed to achieve dissolved O_2 concentrations of approximately 2 mg l^{-1}. Dissolved O_2 concentrations were measured using an oxygen electrode (Yellow Springs Instruments, OH, USA), daily during the first week and every second day for the remaining treatment duration.

There were eight seedlings per treatment combination (NaCl and aeration treatments) in three replicated experiments, (96 seedlings) and the experiment was conducted for four weeks (Figure a.5.1.).

5.2.3. Measurements of stomatal conductance, root hydraulic conductance and electrolyte leakage

Four weeks after the commencement of treatments, six seedlings were randomly harvested from each treatment for stomatal conductance (g_s), shoot fresh weight and root dry weight measurements. Dry weights were determined after freeze-drying for 72 h. Since shoots were all used for the electrolyte leakage measurements, their dry weights could not be determined. The measurements of g_s were conducted on the upper 3-cm of terminal shoots using a steady state poromoter (LI-COR Inc., Lincoln, NE, USA). They were conducted in the growth room between 3 to 4 hours after the start of the photoperiod. The relative humidity inside the cuvette was set at 50%. Needle surface areas were obtained following computer scanning (Sigma Scan 3.0, Jandel Scientific, San Rafael, CA).

Root hydraulic conductance (K_r) was measured using a high pressure flow meter (HPFM) (Dynamax Inc., Houston, TX, USA), as described by Tyree et al. (1995) and expressed in kg s^{-1} MPa^{-1}. Whole intact root systems were connected to the HPFM system through the shoot excised 2 cm above the root collar. Root systems were gradually pressurized to 0.4 MPa to obtain a pressure-flow relationship. Root hydraulic conductivity (L_p) was obtained by dividing the K_r values by root dry weights and expressed as kg s^{-1} MPa^{-1} g^{-1} DW.

To determine the effects of treatments on root and shoot electrolyte leakage, six seedlings were randomly harvested from each treatment and washed with deionized water three times, each time for five minutes. The electrolyte leakage test was conducted as

described by Renault et al. (1998). Briefly, shoot and root samples, each approximately 0.45 g fresh weight (FW), were cut into one-cm segments and placed in tubes containing 10 ml of deionized water. After one-h incubation, the solutions were replaced with 20 ml of deionized water and incubated for 5 h at 50 RPM in an orbital shaker. Electrical conductivities of the solutions were measured with an electrical conductivity meter HI 8033 (Hanna Instruments Inc., Woonsocket, RI, USA). Total electrolytes were obtained by autoclaving the samples at 121°C followed by freezing overnight at –85°C. The samples were then thawed and incubated at room temperature for 5 h. The total electrolyte content of the solutions was measured and electrolyte leakage was expressed as percentage of the total electrolytes.

5.2.4. Analyses of sodium, chloride and non-structural carbohydrates

One, two, three, and four weeks after the commencement of treatments, six plants were randomly selected and rinsed in deionised water. Plants were separated into shoots and roots for sodium, chloride and carbohydrate analyses. Since I did not have sufficient tissues to conduct separate analyses for needles and stems, therefore, needles and stems were combined and treated as shoots. Freeze-dried shoots and roots were plunged into liquid nitrogen and ground to a fine powder prior to extractions.

Chloride was extracted using the hot water extraction technique (Apostol et al. 2002). Briefly, the plant tissue sample (0.05 g) was leached with 10 ml of hot (70-80°C) water. The aqueous mixture was shaken for 20-30 min, and then centrifuged at 2000 RPM for 20 min. This extraction was repeated and both extracts combined and filtered

through a 45-μm Millipore filter before analyzing by ion chromatography (Dionex-300 Series, Dionex Corp., Sunnyvale, CA, USA).

Sodium was analyzed using the ICP-OES method (Vista-RL CCD Simultaneous ICP-OES, Varian Inc., Victoria, Australia) after strong acid (5 % HNO_3) digestion as previously described (Renault et al. 1999).

Carbohydrates were extracted from shoots and roots three times with hot 85% ethanol at 95°C. Soluble carbohydrate (sugar) concentrations were determined colorimetrically using phenolsulfuric acid as described by Smith et al. (1964). Starch was extracted with NaOH and hydrolyzed by an enzyme mixture containing α- amylase (EC 3.2.1.1, ICN 190151, from *Bacillus licheniformis*) and amyloglucosidase (EC 3.2.1.3, Sigma A3514, from *Aspergillus niger*) and incubated for 41 h before the colorimetric measurements with the glucose-oxidase/peroxidase-o-dianisidine reagent (Sigma Glucose Diagnostic Kit 510A) (Haissig and Dickson 1979). Absorbance readings were determined with an Ultrospec III spectrophotometer (Pharmacia LKB, UK) and sugar and starch concentrations were calculated on a dry weight basis.

5.2.5. Statistical analyses

Statistical analyses of the data were performed using SAS general linear model (SAS Institute Inc., Cary, NC 1996). ANOVA were also determined for the treatment effects by two-way interactions between NaCl and aeration treatments. NaCl and aeration treatments were treated as fixed effects. Sodium, chloride, sugar and starch concentrations in plants tissues were analyzed for each treatment week. Correlation

analyses between root electrolyte leakage and root Na^+ or root Cl^- concentrations were performed using the CORR procedure of SAS. A model used in the present study was:

$$Y_{ijk} = \mu + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where:
Y_{ijk} = response variable
μ = overall mean
ρ_i = replication (random factor), i = 1…3;
α_j = sodium chloride effect (fixed factor), j= 1…2;
β_k = aeration effect (fixed factor), k = 1…2 ;
$(\alpha\beta)_{jk}$ = NaCl and aeration interaction effect; and
ε_{ijk} = random error

5.3. Results

5.3.1 Shoot and root weights, stomatal conductance and root water flow

Sodium chloride and aeration treatments did not significantly affect shoot fresh weights (Figure5.1a) but NaCl ($P<0.05$) and hypoxia ($P<0.001$) significantly reduced root dry weights (Figure5.1b).

Stomatal conductance was significantly reduced by NaCl ($P<0.001$) and hypoxia ($P<0.001$), and the interaction of NaCl and aeration treatments on g_s was significant ($P<0.009$) (Figure 5. 2a). Root hydraulic conductance (K_r) was reduced by both salinity ($P<0.001$) and hypoxic ($P<0.001$) treatments. There was a significant interaction ($P<0.001$) between NaCl and aeration treatments on K_r. In the absence of NaCl, hypoxia reduced K_r by 40% while 45 mM NaCl well-aerated plants had K_r value by approximately 50% lower compared with well-aerated control plants (Figure 5. 2b). Plants subjected to NaCl + hypoxia treatment showed a 72 % reduction in K_r compared

with the well-aerated plants treated with NaCl (Figure 5. 2b). Root hydraulic conductivity (L_p) was reduced by salinity ($P<0.005$) and hypoxia ($P<0.01$). However, plants exposed to both hypoxia and salinity showed almost 85% increase in L_p compared with the well-aerated plants treated with salt (Figure 5. 2c).

5.3.2 Electrolyte leakage and salt uptake

Neither the aeration level nor salinity significantly affected needle electrolyte leakage (Figure 5. 3a). Hypoxia induced a significant ($P<0.001$) increase in root electrolyte leakage, while NaCl and its interaction with aeration level showed no significant effect. Mean root electrolyte leakage from plants treated with NaCl under hypoxic conditions increased by about 40 % compared with hypoxic seedlings that were not treated with NaCl (Figure 5. 3b).

Shoot Na^+ concentration in NaCl-treated plants increased over time (Figure 5. 4a). After one week of treatment, shoot Na^+ concentration in hypoxic plants treated with NaCl was significantly ($P<0.0001$) higher compared with well-aerated plants. However, starting at week two until the end of the treatments on week four, hypoxic and well-aerated plants treated with NaCl had similar shoot Na^+ concentrations (Figure 5. 4a). Over the course of the experiment, root Na^+ concentrations were significantly higher in NaCl treated well-aerated plants compared with NaCl-treated hypoxic plants (Figure 5. 4b). In the final, fourth treatment week, mean Na^+ root concentration in well-aerated plants was 8.25 mg g^{-1} DW) compared with 2.26 mg g^{-1} DW measured in hypoxic NaCl-treated plants (Figure 5. 4b).

Similar to Na^+, hypoxia altered shoot Cl^- concentration in NaCl-treated plants after only one week in the treatments (Figure 5. 5a). In NaCl-treated roots, Cl^- concentrations were significantly higher in well-aerated plants than those in hypoxic plants for the three initial weeks (Figure 5. 5b). However, on week four, hypoxic roots contained about 41% more Cl^- compared with well-aerated plants (Figure 5. 5b).

5.3.3. Non-structural carbohydrates

Shoot starch concentrations were similar in all treatments until week 3 (Figure 5. 6a). Four weeks after the initiation of treatments, starch concentrations increased about three-fold in well-aerated plants and two-fold in hypoxic plants compared with the shoots of plants treated with NaCl (Figure 5. 6a). Roots contained relatively lower starch concentrations compared with shoots (Figure 5. 6b). In all treatments, with the exception of well-aerated NaCl-treated plants, root starch concentrations increased four weeks after the start of treatments (Figure 5. 6b).

Similar to starch, shoots in all treatments contained relatively higher concentrations of sugars (Figure 5. 7a) compared with roots (Figure 5. 7b). In both roots and shoots, sugar concentrations were several-fold higher than starch (Figure 5. 7a,b). Shoot sugar concentrations were similar in all treatments throughout the experiment (Figure 5. 7a). In roots, salt-treated well-aerated plants had almost two-fold higher concentrations of sugars compared with other treatments after one week (Figure 5. 7b). There was no significant difference between the treatments on weeks two and three. However, after four weeks of treatment, NaCl-treated well-aerated plants showed 30%

increase in root sugar concentrations compared with NaCl-treated hypoxic plants (Figure 5. 7b).

5.4. Discussion

Over four weeks of treatment, neither salt nor aeration treatments affected shoot fresh weights. However, I observed needle chlorosis, needle and root tip necrosis, and fewer new roots produced in plants grown under hypoxic saline conditions. Both salinity and hypoxia reduced g_s with a concomitant decline in K_r. A decrease in stomatal conductance is frequently observed in salt-treated plants (Renault et al. 2001b), possibly due to the osmotic responses of roots to salinity and resulting effects on root and shoot water relations (Rodriguez et al. 1997). The observed initial high level of soluble sugars in roots of well-aerated plants treated with NaCl (Figure 5. 7b) suggests that the plants accumulated sugars as an initial transient response to salinity. However, since this response did not persist for the remaining treatment duration, its potential significance in stress protection is unclear.

The decrease in g_s of NaCl-treated and hypoxic plants could be due to inhibition of root hydraulic conductance. Reductions in root water flow rates have been previously reported in hypoxic (Kamaluddin and Zwiazek 2002) and salt-treated plants (Azaizeh and Steudle 1991; Rodriguez et al. 1997). These reductions have been attributed to the inhibition of water transport through water channels (Drew et al. 1994; Zhang and Tyerman 1999; Carvajal et al. 2000). In the present study, hypoxia aggravated the effects of salinity on root mortality and growth inhibition, thus decreasing the size and number of functional roots available for water transport. Plants grown under saline conditions

showed reduced root dry weight, which was further reduced in hypoxic plants by almost 50%. Therefore, I also determined L_p values for all plants to account for changes in root size as a result of experimental treatments. The decreases in L_p values suggest that both hypoxia and salt had an inhibitory effect on root tissue water flow properties. However, an opposite effect was observed in hypoxic plants subjected to salt stress, where L_p values increased. This response was likely due to root tissue and root cell membrane injury by the combined treatments and resulting decrease of cell and tissue resistance to water flow by damaged roots, similar to that observed in flooded tomato plants (Else et al. 1995). High electrolyte leakage from hypoxic roots treated with salt support this hypothesis.

Root hydraulic conductance and leaf stomatal conductance are almost simultaneously inhibited by a number of environmental stresses including drought (Dubrovsky et al. 1998; Fotelli et al. 2000), low soil temperature (Wan et al. 1999), hypoxia (Zhang and Tyerman 1991; Kamaluddin and Zwiazek 2002), and metabolic inhibitors (Kamaluddin and Zwiazek 2001). By reducing plant water uptake, both processes likely contribute to a reduction in salt uptake and transport to the shoots (Shennan et al. 1987). On several measurement days, the ratio of Na^+ stored in the roots to that present in the shoots of well-aerated plants was higher compared with hypoxic seedlings suggesting that hypoxic roots lost some capacity for Na^+ storage as a salt resistance mechanism. Jacoby (1965) and Wallace et al. (1965) demonstrated that salt tolerance in *Phaseolus vulgaris* is strongly dependent on the retention of Na^+ in roots and stems and this retention is inhibited by hypoxia. Similarly, Drew and Läuchli (1985) observed that transport of Na^+ to the shoots of *Zea mays* increased when roots were grown under anoxic conditions. In my previous studies (Renault et al. 2001a; 2001b), I

found that shoot Na^+ levels rapidly increased when root Na^+ concentrations reached a certain threshold. Higher NaCl concentrations and longer treatment durations in the present study would have likely resulted in differences in shoot Na^+ concentrations between well-aerated and hypoxic plants. Previous studies showed that the ability of roots to exclude Na^+ from the shoots became less effective at higher NaCl concentrations (Drew and Dikumwin 1985). Therefore, long-term exposure of plants to saline and hypoxic conditions may affect shoot growth and plant survival (Barrett-Lennard 1986).

Root electrolyte leakage increased from the roots of plants mostly due to hypoxia. However, salt treatment aggravated electrolyte leakage from hypoxic roots (Figure 5. 3b). After four weeks, hypoxia also caused greater Cl^- accumulation in salt-treated roots while the opposite was observed for Na^+. Between weeks 3 and 4, root Cl^- of hypoxic plants increased by approximately 5-fold, suggesting a severe loss of Cl^- exclusion mechanism. My results showed that root Cl^- concentration was positively correlated ($r=0.97$) with root electrolyte leakage suggesting that the increase in Cl^- root content may be related to the increase in membrane leakiness. The tissue concentrations of Cl^- were higher than those of Na^+. These results suggest that Cl^- may play a significant role in salt injury to jack pine.

The lack of correlation of root Na^+ concentration with root electrolyte leakage ($r=0.43$) could be explained by more effective compartmentalization of root Na^+ in the vacuoles compared with root Cl^- which tends to accumulate mostly in the cytoplasm and cell walls. Further research on the effects of hypoxia on cell membrane function and its relationship with Na^+ and Cl^- uptake and distribution will need to be carried out to explain the exact mechanisms of hypoxia and salinity effects on jack pine.

Carbohydrates are the primary energy storage compounds and are often associated with tolerance to flooding stress (Kozlowski and Pallardy 1997; Huang 2000). Webb and Armstrong (1983) observed that roots ceased to elongate after several hours of being subjected to oxygen deficient conditions due to low carbohydrate reserves in the root tips. The amount of starch is dependent on the growth and metabolic activities of the roots (Kozlowski and Pallardy 1997). Therefore, an accumulation of root starch observed in hypoxic saline jack pine after four weeks of treatment (Figure 5. 6b) could be related to a reduction in root metabolic activity and, possibly, to inhibited root growth.

In conclusion, my study demonstrated that hypoxia aggravated the inhibitory effects of salinity on g_s and K_r and decreased the ability of roots to store Na^+. Hypoxia interacted with salinity stress by affecting membrane permeability of roots and resulted in increased membrane electrolyte leakage. I suggest that membrane function and respiration-dependent processes were responsible for the decrease in root hydraulic conductance and affected the sequestration of Na^+ and Cl^- in plants. Since my study showed that hypoxia aggravated salinity effects in jack pine, other plant species should be considered for the revegetation of areas where a combination of salt and soil oxygen deficiency are likely to occur.

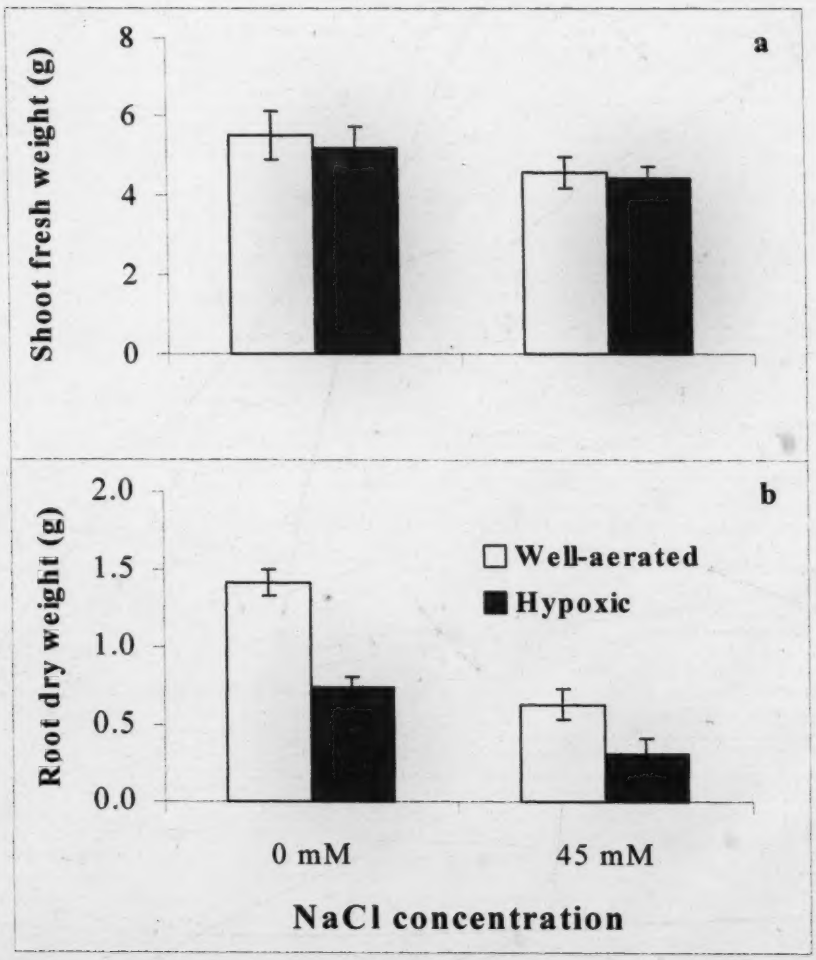

Figure 5. 1. Effects of NaCl and aeration treatments on a) shoot fresh weight, and b) root dry weight in 6-month old jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

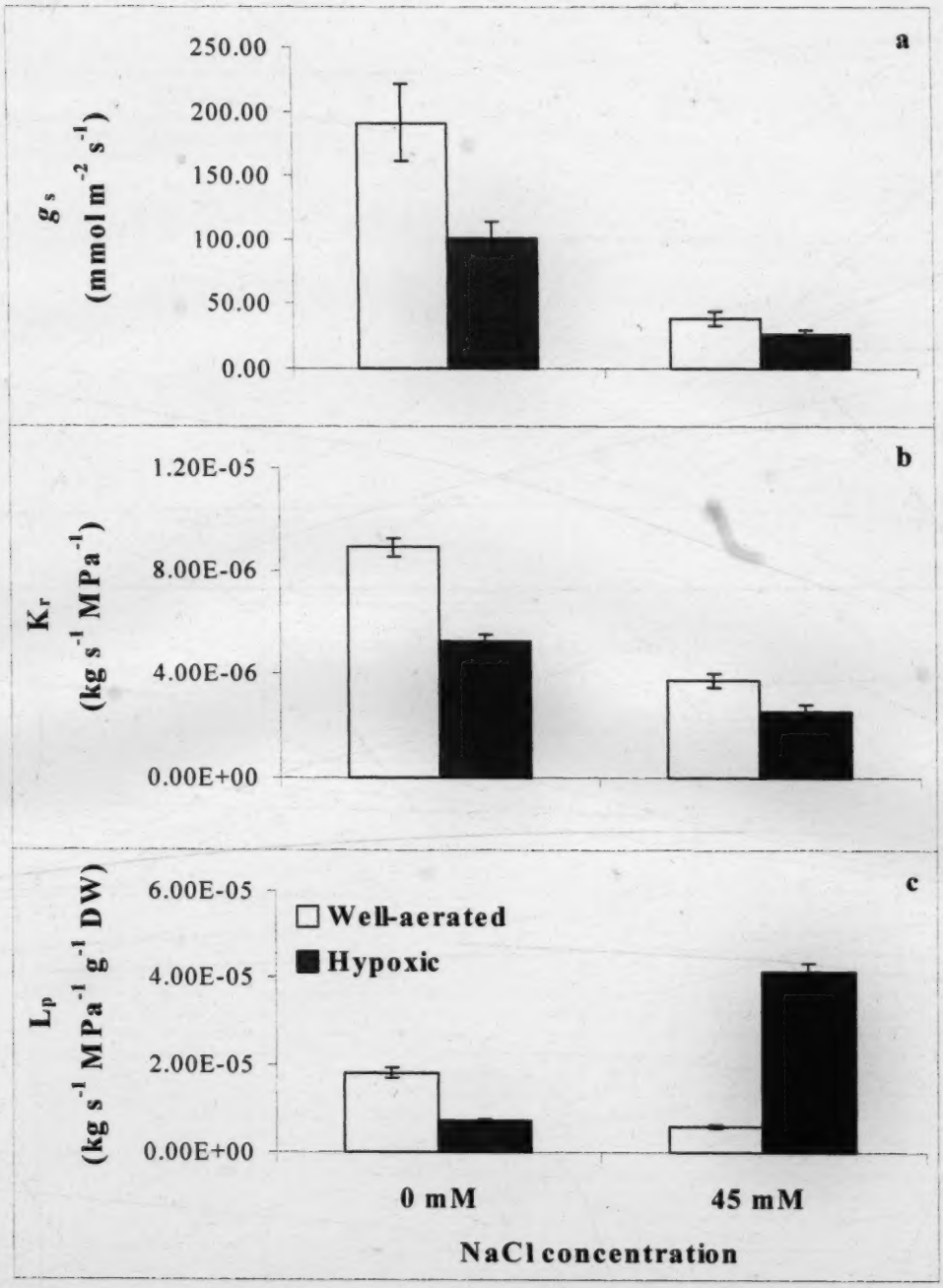

Figure 5. 2. Effects of NaCl and aeration treatments on a) stomatal conductance (g_s) b) root hydraulic conductance (K_r), and c) root hydraulic conductivity (L_p) in 6-month old jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

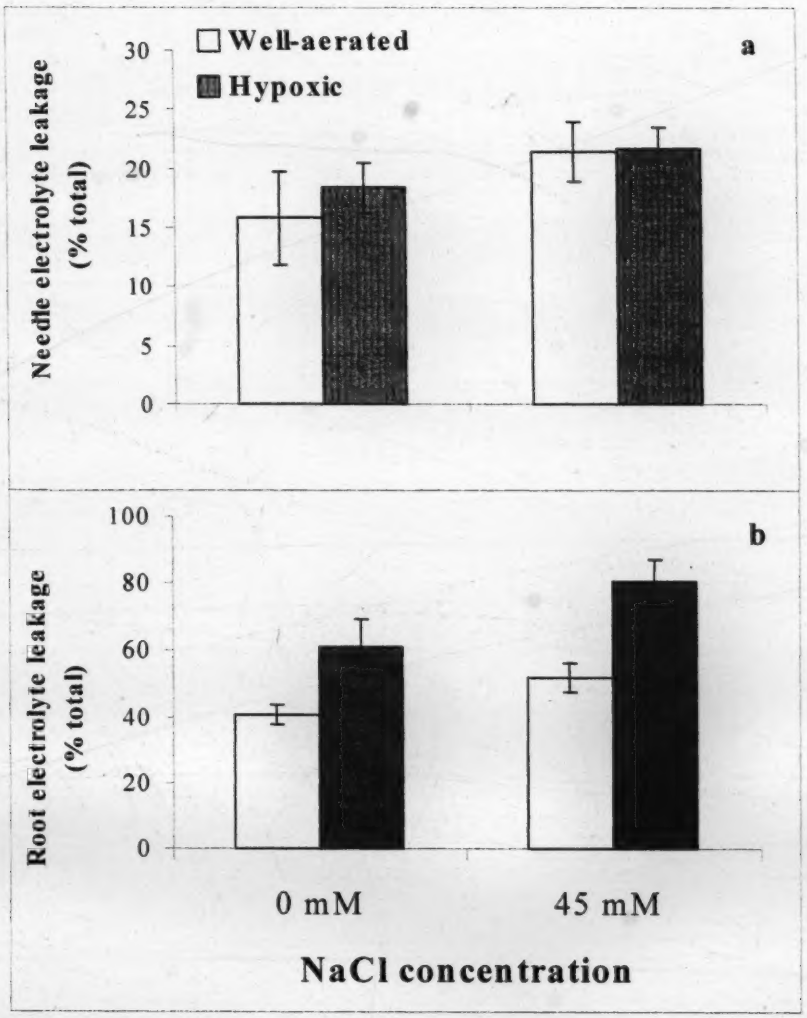

Figure 5. 3. Effects of NaCl and aeration treatments on a) needle electrolyte leakage, and b) root electrolyte leakage in 6-month old jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

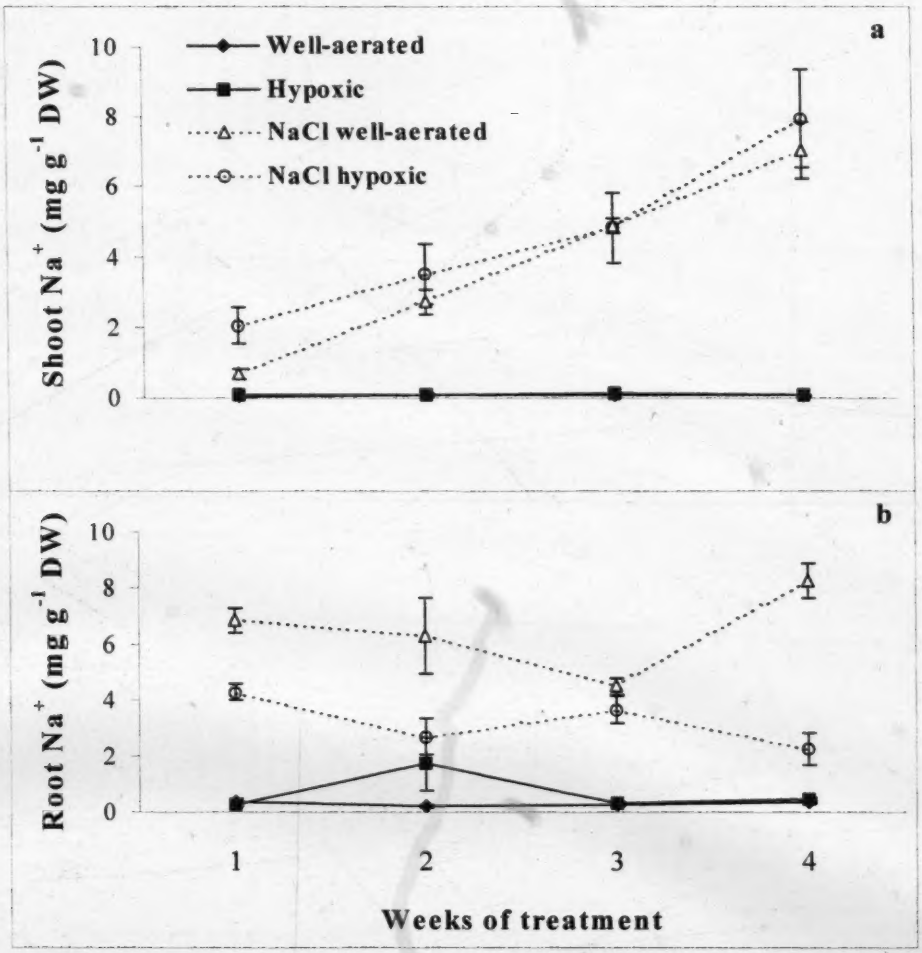

Figure 5. 4. Effects of NaCl and aeration treatments on Na^+ concentrations in a) shoots, and b) roots in 6-month old jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

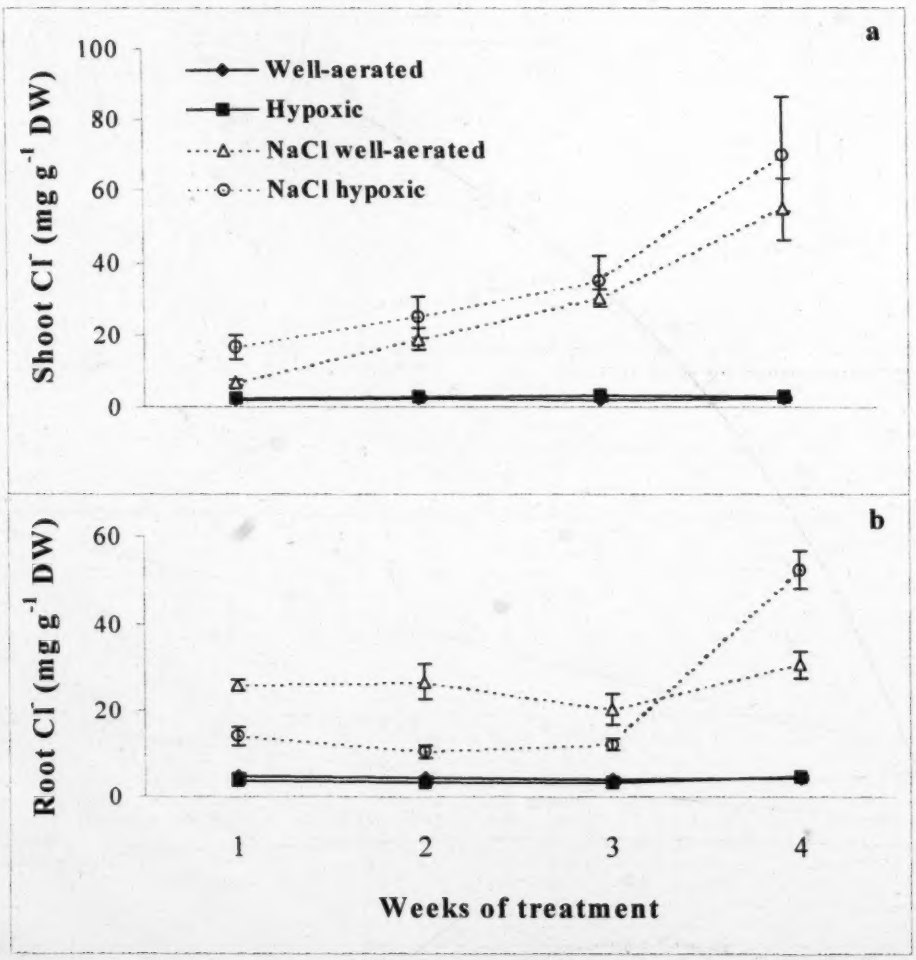

Figure 5. 5. Effects of NaCl and aeration treatments on Cl⁻ concentrations in a) shoots, and b) roots in 6-month old jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

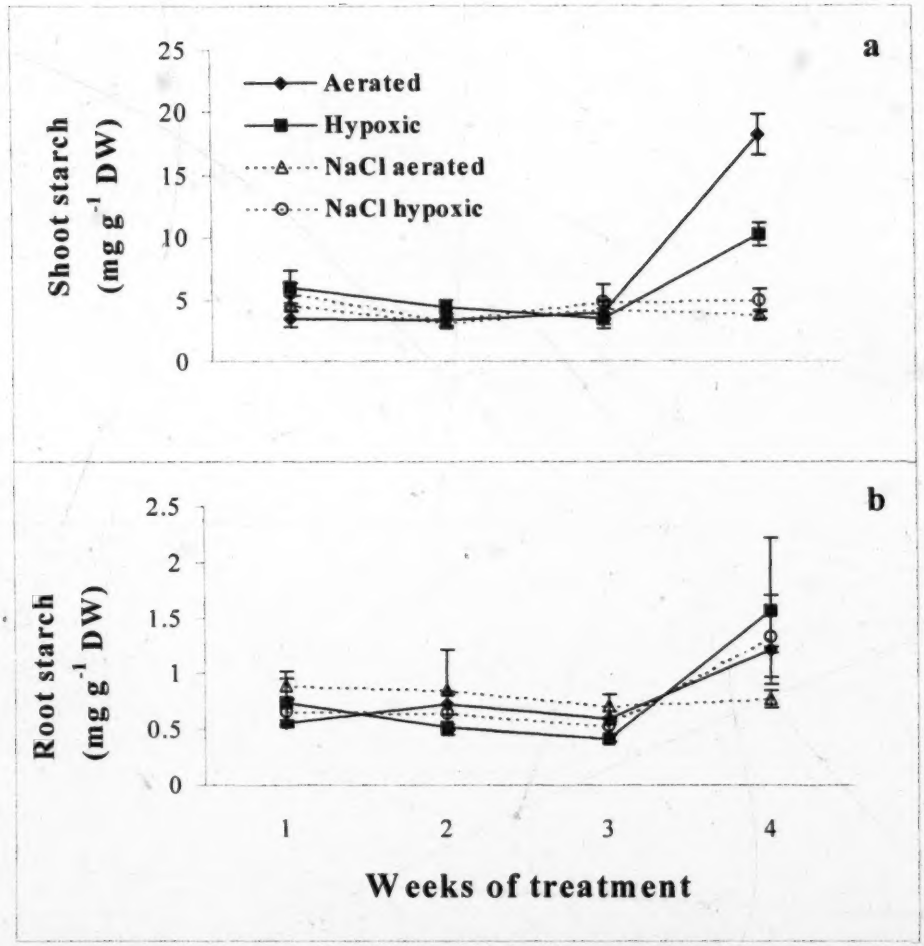

Figure 5. 6. Effects of NaCl and and aeration treatments on starch concentrations in a) shoot and b) root in jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

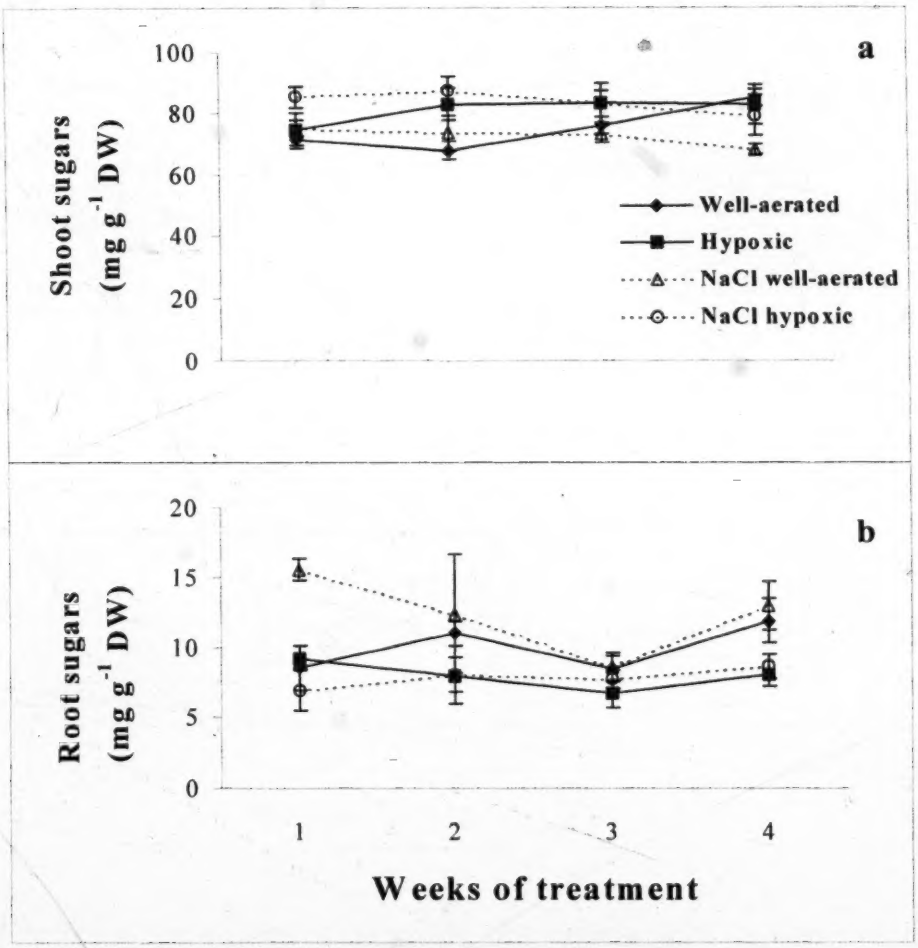

Figure 5. 7. Effects of NaCl and aeration treatments on sugar concentrations in a) shoot and b) root in jack pine grown in solution culture for four weeks. Each data point represents mean (n=6) ± SE.

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5.6 Appendices

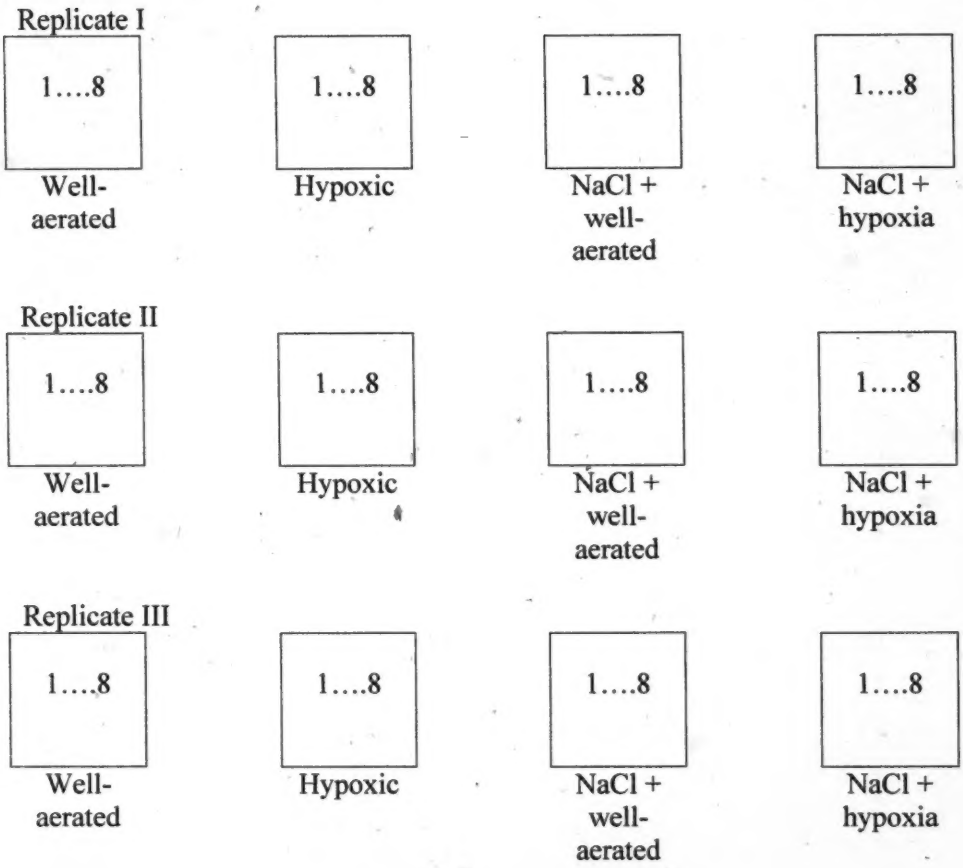

Figure A.5.1. Experimental layout of the study. Number (n=8) represents the number of seedlings placed in each treatment container, which was replicated three times

Table A.5. 1 ANOVA for shoot and root weights, water conductance and tissue electrolyte leakage of jack pine seedlings treated with NaCl (0 and 45 mM) and aeration levels (well-watered and hypoxic), with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr<F
Shoot fresh weight	NaCl	1	1.75	0.2045
	Aeration	1	0.01	0.9409
	Rep	2	0.36	0.7027
	NaCl*Aeration	1	0.16	0.6922
	Error	16		
Root dry weight	NaCl	1	204.99	0.0001
	Aeration	1	140.26	0.0001
	Rep	2	3.09	0.0753
	NaCl*Aeration	1	18.65	0.0006
	Error	16		
Stomatal conductance (g_s)	NaCl	1	47.44	0.0001
	Aeration	1	17.53	0.0006
	Rep	2	1.46	0.2603
	NaCl*Aeration	1	8.60	0.0093
	Error	17		
Root hydraulic conductance (K_r)	NaCl	1	550.58	0.0001
	Aeration	1	207.73	0.0001
	Rep	2	53.86	0.0001
	NaCl*Aeration	1	0.08	0.9248
	Error	17		
Root hydraulic conductivity (L_p)	NaCl	1	11.13	0.0053
	Aeration	1	7.79	0.0143
	Rep	2	0.59	0.6543
	NaCl*Aeration	1	5.43	0.0457
	Error	17		
Needle electrolyte leakage	NaCl	1	0.12	0.7366
	Aeration	1	0.19	0.6725
	Rep	2	1.20	0.3250
	NaCl*Aeration	1	2.43	0.1375
	Error	17		
Root electrolyte leakage	NaCl	1	1.30	0.2708
	Aeration	1	14.39	0.0014
	Rep	2	1.07	0.3664
	NaCl*Aeration	1	2.69	0.1194
	Error	17		

Table A.5. 2 ANOVA for shoot and root Na^+ concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and aeration levels (well-watered and hypoxic) after first, second, third and four week of treatments, with treatment containers replicated (Rep) three times.

Parameter	Week	SV	df	F	Pr<F
Shoot Na^+	1	NaCl	1	25.79	0.0001
		Aeration	1	7.20	0.0152
		Rep	2	0.27	0.7663
		NaCl*Aeration	1	6.91	0.0170
		Error	18		
	2	NaCl	1	48.14	0.0001
		Aeration	1	0.81	0.3804
		Rep	2	0.57	0.5742
		NaCl*Aeration	1	0.87	0.3621
		Error	18		
	3	NaCl	1	99.22	0.0001
		Aeration	1	0.00	0.9797
		Rep	2	0.49	0.6204
		NaCl*Aeration	1	0.01	0.9364
		Error	18		
	4	NaCl	1	102.02	0.0001
		Aeration	1	0.26	0.6145
		Rep	2	1.49	0.2540
		NaCl*Aeration	1	0.27	0.6075
		Error	18		
Root Na^+	1	NaCl	1	447.67	0.0001
		Aeration	1	28.55	0.0001
		Rep	2	1.47	0.2567
		NaCl*Aeration	1	24.31	0.0001
		Error	18		
	2	NaCl	1	16.88	0.0007
		Aeration	1	1.45	0.2449
		Rep	2	1.32	0.2912
		NaCl*Aeration	1	8.75	0.0084
		Error	18		
	3	NaCl	1	203.75	0.0001
		Aeration	1	1.98	0.1762
		Rep	2	0.22	0.8081
		NaCl*Aeration	1	2.97	0.1019
		Error	18		
	4	NaCl	1	133.87	0.0001
		Aeration	1	48.31	0.0001
		Rep	2	0.62	0.5516
		NaCl*Aeration	1	51.80	0.0001
		Error	18		

Table A.5. 3 ANOVA for shoot and root Cl^- concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and aeration levels (well-watered and hypoxic) after first, second, third and four week of treatments, with treatment containers replicated (Rep) three times.

Parameter	Week	SV	df	F	Pr<F
Shoot Cl^-	1	NaCl	1	32.34	0.0001
		Aeration	1	9.13	0.0073
		Rep	2	0.87	0.4355
		NaCl*Aeration	1	8.46	0.0094
		Error	18		
	2	NaCl	1	44.58	0.0001
		Aeration	1	1.27	0.2755
		Rep	2	1.70	0.2101
		NaCl*Aeration	1	0.96	0.3401
		Error	18		
	3	NaCl	1	76.54	0.0001
		Aeration	1	0.68	0.4216
		Rep	2	1.20	0.3228
		NaCl*Aeration	1	0.28	0.6059
		Error	18		
	4	NaCl	1	54.26	0.0001
		Aeration	1	0.83	0.3759
		Rep	2	1.59	0.2321
		NaCl*Aeration	1	0.74	0.4021
		Error	18		
Root Cl^-	1	NaCl	1	158.65	0.0001
		Aeration	1	27.12	0.0001
		Rep	2	0.29	0.7523
		NaCl*Aeration	1	18.94	0.0004
		Error	18		
	2	NaCl	1	42.85	0.0001
		Aeration	1	14.71	0.0012
		Rep	2	0.15	0.8598
		NaCl*Aeration	1	11.36	0.0034
		Error	18		
	3	NaCl	1	47.62	0.0001
		Aeration	1	6.34	0.0221
		Rep	2	2.29	0.1317
		NaCl*Aeration	1	3.64	0.0734
		Error	18		
	4	NaCl	1	5.17	0.0363
		Aeration	1	0.60	0.4507
		Rep	2	1.74	0.2046
		NaCl*Aeration	1	0.56	0.4654
		Error	18		

Table A.5. 4 ANOVA for shoot and root starch concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and aeration levels (well-watered and hypoxic) after first, second, third and four week of treatments, with treatment containers replicated (Rep) three times.

Parameter	Week	SV	df	F	Pr<F
Shoot starch	1	NaCl	1	0.05	0.8232
		Aeration	1	2.80	0.1118
		Rep	2	0.07	0.9328
		NaCl*Aeration	1	0.45	0.5094
		Error	18		
	2	NaCl	1	0.86	0.3650
		Aeration	1	1.59	0.2231
		Rep	2	1.46	0.2590
		NaCl*Aeration	1	0.68	0.4209
		Error	18		
	3	NaCl	1	0.60	0.4478
		Aeration	1	0.09	0.7625
		Rep	2	0.97	0.3969
		NaCl*Aeration	1	0.18	0.6747
		Error	18		
	4	NaCl	1	3.63	0.0739
		Aeration	1	0.31	0.5825
		Rep	2	0.55	0.5888
		NaCl*Aeration	1	0.80	0.3846
		Error	18		
Root starch	1	NaCl	1	1.11	0.3066
		Aeration	1	2.55	0.1289
		Rep	2	0.02	0.9810
		NaCl*Aeration	1	2.99	0.1017
		Error	18		
	2	NaCl	1	0.58	0.4554
		Aeration	1	0.35	0.5627
		Rep	2	1.08	0.3603
		NaCl*Aeration	1	0.96	0.3413
		Error	18		
	3	NaCl	1	2.17	0.1583
		Aeration	1	7.73	0.0124
		Rep	2	0.54	0.5918
		NaCl*Aeration	1	0.00	0.9571
		Error	18		
	4	NaCl	1	0.71	0.4113
		Aeration	1	1.41	0.2511
		Rep	2	1.75	0.2036
		NaCl*Aeration	1	0.22	0.6472
		Error	18		

Table A.5. 5 ANOVA for shoot and root sugars concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and aeration levels (well-watered and hypoxic) after first, second, third and four week of treatments, with treatment containers replicated (Rep) three times.

Parameter	Week	SV	df	F	Pr<F
Shoot sugars	1	NaCl	1	1.93	0.1822
		Aeration	1	3.70	0.0705
		Rep	2	1.72	0.2068
		NaCl*Aeration	1	2.48	0.1326
		Error	18		
	2	NaCl	1	2.49	0.1321
		Aeration	1	8.69	0.0086
		Rep	2	1.77	0.1994
		NaCl*Aeration	1	0.01	0.9363
		Error	18		
	3	NaCl	1	0.41	0.5285
		Aeration	1	5.91	0.0257
		Rep	2	0.64	0.5366
		NaCl*Aeration	1	0.08	0.7762
		Error	18		
	4	NaCl	1	6.14	0.0240
		Aeration	1	0.35	0.5645
		Rep	2	0.69	0.5153
		NaCl*Aeration	1	1.69	0.2109
		Error	18		
Root sugars	1	NaCl	1	2.79	0.1131
		Aeration	1	12.57	0.0025
		Rep	2	2.11	0.1521
		NaCl*Aeration	1	8.88	0.0084
		Error	18		
	2	NaCl	1	0.06	0.8061
		Aeration	1	2.62	0.1228
		Rep	2	0.85	0.4436
		NaCl*Aeration	1	0.14	0.7163
		Error	18		
	3	NaCl	1	0.20	0.6604
		Aeration	1	2.50	0.1314
		Rep	2	0.16	0.8503
		NaCl*Aeration	1	0.35	0.5636
		Error	18		
	4	NaCl	1	0.24	0.6336
		Aeration	1	8.71	0.0089
		Rep	2	1.65	0.2208
		NaCl*Aeration	1	0.01	0.9209
		Error	18		

CHAPTER SIX

Naphthenic acids affect plant water conductance but do not alter shoot Na^+ and Cl^- concentrations in jack pine (*Pinus banksiana*) seedlings

6.1. Introduction

Naphthenic acids (NAs) are complex mixtures of mono- and polycyclic alkanes that contain carboxylated aliphatic side chains of various lengths (Fan 1991). NAs are used in wood preservation industry (Brient et al. 1995), as emulsifying agents in the production of agricultural insecticides and as additives and emulsion breakers in the oil industry (Hatch and Matar 1977). NAs are natural constituents in nearly all crude oils (Brient et al. 1995) and are present as sodium salts in the oil sands in western Canada (Schramm et al. 2000). The oil sands industry in northeastern Alberta extracts light crude oil with hot caustic water, which generates large volumes of tailings that are relatively alkaline, with a pH of > 8.0, and contain NAsat concentrations ranging from 50 – 100 mg l^{-1} (MacKinnon and Boerger 1986). However, it has been reported that soil concentrations in many oil sands reclamation sites may contain NAs concentrations higher than 100 mg l^{-1} as a result of evaporation (Kamaluddin and Zwiazek 2002).

Despite the commercial use of NAs and their presence in reclamation areas, little is known about the potential impacts of NAs on plants. NAs have been reported to cause injury to aquatic organisms (MacKinnon and Boerger 1986; CONRAD 1998). A recent study also showed that NAs inhibited leaf growth, stomatal conductance and net photosynthesis in aspen (*Populus tremuloides*) seedlings (Kamaluddin and Zwiazek 2002). These effects were thought to be due to the surfactant properties of NAs

(Kamaluddin and Zwiazek 2002). Surfactants can disrupt membrane integrity (Quinn 1976) and similarly to other hydrocarbons, they can displace membrane lipids (van Overbeek and Blondeau, 1954).

In addition to elevated levels of NAs, oil sands reclamation areas are also relatively saline with an electrical conductivity of > 4 dS m^{-1} (MacKinnon et al. 2000), which is considered detrimental to plants (Singer and Munns 1996; Renault et al. 1998). Since salinity and NAs inhibit water uptake and reduce plant growth (Munns and Termaat 1986; Kamaluddin and Zwiazek, 2002), NAs may aggravate salinity problems with plant water uptake in salt-affected reclamation areas.

Salinity can have dramatic effects on plant water relations (Bolanos and Longstretch 1984). NaCl was found to decrease root hydraulic conductivity of *Zea mays* (Aizazeh et al. 1992; Evlagon et al. 1992), which could potentially affect transpiration rates. Transpiration was demonstrated to influence the rate of ion transport to the shoot and the accumulation of ions in several salt-stressed plants (Lauter and Munns 1987; Salim 1989). The injury observed in plants exposed to salt is often correlated with reduced ability of roots to store Na^+ and Cl^- and with the accumulation of Na^+ and Cl^- in the shoots (Chapter Five, Yeo et al. 1977; Grieve and Walker 1983). This accumulation has been explained as partly due to the injury of root cell membranes (Kuiper 1968; Mansour 1997). Since NAs are chemically related to fatty acids (Brient et al. 1995), it is possible that they could be incorporated into the cell membranes and affect cell membrane permeability.

In the present study, I examined the hypothesis that NAs exacerbate inhibition of water conductance to shoots and membrane leakiness caused by NaCl and increase salt

uptake in jack pine (*Pinus banksiana* Lamb.) seedlings. I selected jack pine for this study due to its relative susceptibility to salt (Renault et al. 1998; Apostol et al. 2002) and its importance for the reclamation of oil sands in northeastern Alberta.

6.2. Materials and methods

6.2.1 Plant material and growth conditions

Jack pine (*Pinus banksiana* Lamb.) seeds were collected from trees approximately 60 km north of Ft. McMurray, Alberta (57°05.95N 111°38.90W). The seeds were germinated and seedlings grown for six months in Spencer-Lemaire root trainers (350 ml volume, Steuwe and Sons, Inc., Corvallis, OR, USA) filled with a mixture of peat moss and coarse washed sand (2:1, v/v). The seedlings were grown in a growth room under the following environmental conditions: day/night temperature, 22/18°C; relative humidity, approximately 70%; 18-h photoperiod, and photosynthetic photon flux density of approximately 300 μmol m^{-2} s^{-1} at the seedling level. After 6 months, roots were rinsed in water and the seedlings were transferred to 10-l containers filled with aerated mineral nutrient solution as previously described (Apostol et al. 2002). Seventy- two (72) seedlings were grown in solution culture for two weeks before the commencement of treatments and the solution was replaced weekly.

6.2.2 Sodium chloride and naphthenic acids treatments

Salinity treatment was imposed by adding 15 mM NaCl to the nutrient solution daily for three days, to reach a final concentration of 45 mM NaCl. Naphthenic acids were prepared by mixing stock solution of sodium salt of NAs (ACROS Organics, NJ, USA) with nutrient solution until the desired concentration of 150 mg NAs l^{-1} was achieved. NaOH was added to adjust the pH of each treatment and control nutrient solutions to 7.8 to avoid NAs precipitation (Kamaluddin and Zwiazek 2002).

The treatments were arranged in a 2 x 2 (two levels of NaCl; 0 and 45 mM NaCl and two levels of NAs; 0 and 150 mg l^{-1}) factorial randomized design. Six seedlings were placed in each container, in three replicated containers with 18 seedlings per treatment. A total of 72 seedlings were treated for four weeks (Figure A.6.1).

6.2.3. Growth, stomatal conductance (g_s) and root hydraulic conductance (K_r)

After four weeks of treatments, six seedlings from each treatment were randomly harvested for fresh and dry weight measurements. Dry weight was obtained after freeze-drying the samples for 72 h. Stomatal conductance (g_s) measurements were made on the other randomly selected seedlings between 3 and 4 h after the onset of the photoperiod. The measurements were conducted on the upper 3-cm portion of the shoot using a steady-state porometer LI-600 (Li-Cor Inc., NE, USA) and expressed on the needle area basis following computer scanning (Sigma Scan 3.0, Jandel Scientific, San Rafael, CA, USA).

Root hydraulic conductance (K_r) was measured on the same six seedlings used for stomatal conductance measurements using a high pressure flow meter (HPFM) (Dynamax Inc., Houston, TX, USA), as described by Tyree et al. (1995) and expressed in kg s^{-1} MPa^{-1}. Whole intact root systems, immersed in either control or treatment solutions, were connected to the HPFM system through the shoot excised 2 cm above the root collar. Root systems were gradually pressurized to 0.4 MPa to obtain a pressure-flow relationship.

6.2.4. Root respiration

Root respiration was measured as oxygen uptake using an oxygen electrode (Yellow Springs Instruments, Yellow Spring, OH, USA). Intact roots were placed in a 250-ml airtight cuvette filled with aerated treatment or control solutions that were continuously stirred with a magnetic stirrer. Root respiration was monitored for 20 minutes by recording the oxygen uptake every 2 min. Root respiration rates were calculated as a mean of oxygen uptake over time and values were expressed in mmol O_2 root $system^{-1}$ min^{-1}.

6.2.5. Measurements of tissue electrolyte leakage

Prior to the electrolyte leakage test, shoots and roots were washed three times with deionized water, each time for five minutes. The electrolyte leakage test was conducted as described by Apostol et al. (2002). Briefly, shoot and root samples, each

approximately 0.45 g fresh weight (FW), were placed in tubes containing deionized water and incubated for 5 h. Electrical conductivities of the solutions (EC_L) were measured with an electrical conductivity meter HI 8033 (Hanna Instruments Inc., Woonsocket, RI, USA). Total electrolytes (EC_T) were obtained by autoclaving the samples at 121°C followed by freezing overnight at -85°C and thawing at room temperature (23 °C) for 5 h. Electrolyte leakage was calculated as the percentage of total electrolytes in the solution after 5 h ($EC_L/EC_T * 100$).

6.2.6. Osmotic potential and naphthenic acids (NAs) determination

Xylem saps were collected for osmotic potential and NAs determinations. The xylem saps were collected from the roots of five seedlings that were immersed in the respective treatment and control solutions. Intact roots were placed in a pressure chamber and pressurized to 0.3 MPa for 1 h. Osmotic potential of the xylem sap was measured with a thermocouple psychrometer (HR-33T, 5112, Wescor, Logan, UT, USA) and a C52 sensor in the dew point mode. Xylem saps obtained from the pressurized roots were analysed for NAs using a Syncrude Canada Ltd. Method based on Fourier Transform Infrared (FT-IR) analysis of methylene chloride (CH_2Cl_2) extracts of acidified (pH 2.5) samples (Jivraj et al. 1996).

6.2.7. Ion analyses of plant tissues and xylem saps

Shoots and roots, which were not used for the electrolyte leakage test, were used for ion analyses. Freeze-dried shoots and roots were weighed and ground in liquid nitrogen to a fine powder prior to extractions. Sodium, potassium, and magnesium contents were quantified using the ICP-OES method (Vista-RL CCD Simultaneous ICP-OES, Varian Inc., Victoria, Australia) after strong acid (5 % HNO_3) digestion as previously described (Renault et al. 1999). Chloride and sulfate were extracted using the hot water extraction technique as described earlier (Apostol et al. 2002) and chloride content was determined by ion chromatography (Dionex-300 Series, Dionex Corp., Sunnyvale, CA, USA). Tissue Total N concentrations were determined colorimetrically using the Technicon AutoAnalyzer II (Technicon Industrial Systems, Tarrotown, NY) after digestion with H_2SO_4 and H_2O_2 at 350°C (Richards 1993). Ion analyses in the xylem sap were carried out after digesting diluted sap samples in 0.1 M HNO_3 using the techniques described for plant tissues.

6.2.8 Experimental design and statistical analysis

Analysis of variance (ANOVA) and correlation analyses were performed using SAS GLM (General Linear Model) (SAS Institute Inc., Cary, NC, USA). ANOVA were determined for the treatment effects by two-way interactions between NaCl and NAs treatments. Below is the model used in the present study:

$$Y_{ijk} = \mu + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}$$

where:
Y_{ijk} = response variable
μ = overall mean
ρ_i = replication (random factor), i = 1..3;
α_j = sodium chloride effect (fixed factor), j = 1...2;
β_k = naphthenic acids effect (fixed factor); k = 1..2
$(\alpha\beta)_{jk}$ = NaCl and NAs treatment interaction effect; and
ε_{ijk} = random error

6.3. Results

Shoot fresh weight (Table 6. 1) was significantly inhibited by NaCl ($P<0.0218$), but not by NAs treatments ($P<0.2254$). There were no significant effects of NaCl and NAs treatments on root fresh weights and no significant interaction effects between salinity and NAs on shoot ($P<0.7911$) and root ($P<0.7733$) fresh weights.

Stomatal conductance was significantly reduced by NaCl ($P<0.0001$) and NAs ($P<0.0014$) treatments (Figure 6. 1a). There was a significant ($P<0.0012$) interaction effect between NaCl and NAs on g_s. Addition of NAs to plants treated with NaCl significantly reduced mean g_s values by 33 % compared with plants treated with NaCl treatment alone. Both NaCl ($P<0.0001$) and NAs ($P<0.0001$) significantly inhibited K_r (Figure 6. 1b). A significant ($P<0.0005$) interaction effect between NaCl and NAs was observed for K_r. Mean K_r of plants treated with NaCl alone was reduced by 73% while K_r of NAs-treated plants was 75% lower compared with control plants. When NAs were added to NaCl treatment, plants showed almost 90% reduction in K_r compared with control plants.

When applied individually, NaCl ($P<0.0105$) and NAs ($P<0.0417$) significantly reduced root respiration rate but the interaction effect between NaCl and NAs was not

significant (P<0.5029) (Figure 6. 2a). Respiration rates showed significantly lower values and measured 0.61 mmol O_2 root system^{-1} min^{-1} in NaCl-treated plants and 0.76 mmol O_2 root system^{-1} min^{-1} in NAs-treated plants compared with 1.33 mmol O_2 root system^{-1} min^{-1} in control plants.

Needle electrolyte leakage significantly increased in response to NaCl (P<0.0005) and NAs (P<0.0262) treatments but their interaction effect was not significant (P<0.1275) (Figure 6. 2b). Needle electrolyte leakage from plants treated with NaCl showed 51% increase while NAs-treated plants had 23% increase compared with the control plants. Results of the ANOVA showed that neither NaCl nor NAs treatments significantly affected root electrolyte leakage (Figure 6. 2c).

Shoot Na^+ concentrations of NaCl-treated plants were higher in NaCl-treated plants compared to plants treated with both NaCl and NAs (Figure 6. 3a.). The addition of NAs significantly (P< 0.0065) reduced shoot Na^+ concentrations by almost 40 %. Sodium concentrations of roots in NaCl-treated plants and in NaCl + NAs-treated plants were similar (Figure 6.3b). As for Na^+, plants treated with only NaCl had higher shoot Cl^- concentrations than those treated with NaCl + NAs (Figure 6.4a). Shoots contained about 40 % less Cl^- when plants were grown treated with NAs + NaCl compared with NaCl alone. Root Cl^- concentrations were not significantly altered by NAs (P<0.0042) (Figure 6.4b).

NaCl significantly reduced root K^+ (P<0.0352) and Mg^{2+} (P<0.0032) concentrations while the shoot concentrations were unaffected (Table 6.2). Neither NaCl nor NAs treatments did not significantly affected shoot and root N concentrations and the

interactions between NaCl and NAs were not significant (Table 6.2). Root SO_4^{2-} concentrations significantly increased ($P<0.0418$) in plants treated with NAs (Table 6.2).

Results of short, 1-h exposure of jack pine to NaCl and NAs treatments showed that Na^+ and Cl^- concentrations in the xylem sap were higher in NaCl-treated plants compared with the control plants, with Cl^- concentrations several fold higher than those of Na^+ (Table 6.3). NAs did not appear to alter the concentrations of Na^+ and Cl^- in the xylem sap. Mean osmotic potential values of -0.302 MPa were measured in the xylem sap of plants treated with NaCl compared with -0.453 MPa in NaCl + NAs-treated plants. Mean naphthenic acids concentrations in the xylem sap were approximately 28.60 ± 4.71 mg l^{-1} in NAs-treated plants compared with 84.11± 16.56 mg l^{-1} in NAs + NaCl treatments (Table 6.3).

6.4. Discussion

After four weeks of NaCl treatments, I observed a significant reduction in shoot fresh weight but root fresh weight was not affected. An accumulation of Na^+ and Cl^-, which was observed in the present study, has been reported to be the cause of growth inhibition in several woody plants (Bañuls and Primo-Millo 1992). Roots of NaCl + NAs-treated plants showed extensive necrosis and absence of fine white root tips after four weeks of treatments. Since the plants were grown in relatively high pH (7.8), slight yellowing of needles was observed in all control plants. High pH has been reported to cause leaf chlorosis in *Sheperdia canadensis* and *Populus deltoides* x *P. balsamifera* (Renault et al. 1999) and growth reduction in *Zea mays*, *Triticum aestivum*, *Manihot esculenta*. These effects were associated with nutrient uptake problems (Islam et al. 1980; Noggle and

Fritz 1983). Needle chlorosis and some needle tip necrosis were present in seedlings exposed to NaCl + NAs treatments.

Stomatal conductance was reduced in plants treated with NAs by 80% compared with the control plants. When NAs were combined with NaCl treatment, g_s was further reduced. A similar response was also observed in root hydraulic conductance where NAs increased the inhibition triggered by NaCl. It is possible that the decrease in g_s of salt-treated plants in the presence of NAs could be partly linked to reduction in K_r. The reduction in g_s has been associated with a decline in K_r in salt-treated tomato plants (*Lycopersicon esculentum*) (Rodriguez et al. 1997). Naphthenic acids are surfactants and their surfactant properties could partly explain the reduction in root water uptake (Kamaluddin and Zwiazek 2002). However, I am not certain of their exact effects on water flow characteristics. It has been reported that under environmental stress conditions, hydraulic conductivity of root cell membranes may be reduced due to closure of water channels (Steudle 2000), occlusion of xylem vessels (Byrne et al. 1977; Singh and Sale 2000) and reduction of xylem vessels (Huang et al. 1994). O'Leary (1969) and Zekri and Parsons (1989) demonstrated the decrease in root hydraulic conductivity in salt-treated plants as an effect of root suberization. The exact mechanisms by which NAs reduced K_r of salt-treated plants cannot be determined based on the data obtained in the present study. Further studies will be needed to examine the effects of NaCl + NAs on root anatomy.

Despite the lower Na^+ and Cl^- concentrations in the shoots of NaCl + NA-treated plants compared to NaCl-treated seedlings, I observed more severe wilting in NaCl + NA-treated plants. Therefore, it is plausible that osmotic stress could be partly

responsible for this effect in NaCl + NA- treated plants. Contrary to my hypothesis, my results showed that the shoots of plants treated with NAs contained lower Na^+ and Cl^- concentrations compared with plants treated with NaCl alone (Figure 6. 3 and 6.4). This response could be partly due to the inhibitory effects of NAs on transpiration where a reduction in transpiration rates led to reduced accumulation of salts in the shoots. This is supported by Figure 6. 1, where NAs induced stomatal closure of NaCl-treated plants suggesting that NAs reduced salt uptake through the reduction in transpiration rates. In the present study, NAs reduced K_r of NaCl-treated plants and, therefore, the rate of salt transport to the shoots was likely reduced compared with those plants exposed only to NaCl. Moya et al. (1999) suggested that in citrus species root Cl^- uptake and leaf Cl^- accumulation are linked to water absorption and transpiration rates, respectively. However, these results are contrary to those of (Storey 1995), who suggested that Na^+ and Cl^- transport is largely independent of transpiration rates in salt-tolerant Rangpur lime (*Citrus reticulata* var. *austera*). More recently, Franklin (2002) also showed that transpiration rates were not correlated with Na uptake in jack pine. The lack of effect of transpiration on ion uptake may be partly due to lower transpiration rates in salt-stressed plants (Munns 1985). Clearly, more work is needed to determine the contribution of water conductance in the regulation of Na^+ and Cl^- uptake and transport in plants.

In my previous studies (Apostol et al. 2002; Apostol and Zwiazek 2002), I demonstrated that the injury observed in NaCl-treated jack pine was mainly due to Cl^- accumulation. However, in the present study, I have found significant correlations between needle electrolyte leakage and shoot Na^+ (0.55; $P<0.0265$) and Cl^- (0.60; $P<0.0128$) concentrations. These results suggest that the detrimental effects of NaCl in

jack pine were not only due to the action of Na^+ but also to the negative effects of Cl^-. Martin and Koebner (1995) proposed that both Na^+ and Cl^- contribute to salt toxicity in wheat. Sodium alters cell membrane structure and composition probably by increasing membrane lipid fluidity and reducing its packing density (Mansour 1997). Chloride has also been found to alter membrane lipid composition resulting in increased membrane permeability (Kuiper 1968). Mansour (1997) suggested that when membrane permeability is drastically altered, cell death follows shortly, and this is due to the loss of membrane ability to control cell transport processes. The lack of significant effects of NaCl and NAs treatments on root electrolyte leakage four weeks after initiation of treatments suggests that most of the ions may have leaked out of the membrane resulting in no significant effects of NaCl and NAs.

Recent studies showed that both NaCl (Carvajal et al. 2000) and NAs (Kamaluddin and Zwiazek 2002) inhibited water channel activity resulting in the decline of root water uptake. Kamaluddin and Zwiazek (2002) showed that reduced activity of water channels was reflected by a decline in root respiration. However, I did not observe significant interaction effect between NaCl and NAs treatments on root respiration after 4 weeks of treatment. It is possible that significant interaction effects between NaCl and NAs on root respiration may have been observed earlier. However, the result of the present study may suggest that the effects of NaCl on root respiration dominated the interactive effects of NaCl and NAs and the high variability of seedling responses produced statistically insignificant results. I offer the same explanation for the insignificant interaction effect between NaCl and NAs treatments on needle electrolyte leakage.

The reason for the lack of significant differences in root growth between control plants and treated plants could be due to the effects of pH. It is possible that similar to lupin (Tang et al. 1993), high pH may have inhibited root growth before the effects of NaCl and NAs were observed. Also, it cannot be excluded that longer treatment duration could have produced greater effects on root growth. The slight difference recorded in root Na^+ and Cl^- concentrations between NaCl-treated plants and NaCl + NAs-treated plants may suggest that high NaCl treatment concentration and high pH may have resulted in roots being unable to store Na^+ and Cl^-. Therefore, addition of NAs in the NaCl treatment did not appear to contribute to the accumulation of Na^+ and Cl^- in the roots after four weeks of treatment.

Little is known about the physiological processes involved in nutrient uptake of plants exposed to NAs. Since naphthenic acids have surfactant properties, they may alter root nutrient uptake (Parr and Norman, 1965; Kamaluddin and Zwiazek 2002). I have seen slight reductions in the concentrations of the total root N, K^+ and Mg^{2+} in NaCl + NA-treated plants compared with NaCl-treated plants. Although the differences were not statistically significant, it cannot be excluded that longer treatment durations could have resulted in greater differences in nutrients status between plants exposed to NaCl and NAs treatments and to those exposed to NaCl alone. I also observed an increase in root SO_4^{2-} concentrations in NAs-treated plants but the mechanism of increased SO_4^{2-} concentrations is unclear. Clearly, more work, will be needed to address the effects of NAs on nutrient uptake in plants exposed to NaCl.

In the present study, the higher concentrations of Cl^- compared with Na^+ in the xylem sap reflect greater delivery of Cl^- to the shoots, and subsequently a higher

accumulation of Cl^- than Na^+ in the shoots was observed after, 4-week exposure to NaCl (Figure 6. 3 & 6.4). This also suggests that roots of jack pine had lower capacity to store Cl^- than Na^+. At high external Cl^- concentrations and relatively low cytoplasmic concentrations, it is possible for the membrane potential to be less negative than the Cl^- equilibrium potential leading to passive Cl^- uptake into roots (Tyerman and Skerrett 1999). The low (more negative) value of osmotic potential observed in the xylem sap of plants exposed to both NaCl and NAs treatments paralleled the accumulation of K^+ ((Table 6.3). To date, there have been no reliable methods developed to measure NAs concentrations in plant tissues. This is largely due to the interference from other tissue organic compounds in the NAs analysis. Therefore, the analysis of NAs in xylem sap offers an alternative method of estimating NAs uptake by plants and their transport to shoots. I have shown that NAs were absorbed and transported into the xylem stream through roots and that NaCl appeared to induce NAs uptake in plants (Table 6.3). It is possible that under initial salinity stress conditions; plants immediate response is to take up water in order to maintain a positive water balance. The enhancement of NAs uptake by NaCl-treated plants may be at least partly related to the increased in water uptake. Further work is needed to confirm this hypothesis.

I conclude that the mechanism of NAs action in NaCl-treated jack pine appears to be linked to water transport and that the reduction of water uptake and plant water water flow was partly responsible for a reduction in Na^+ and Cl^- transport to shoots. Further studies should be designed to examine the exact mechanisms by which NAs affect membrane function and modify water, salt and nutrient uptake and distribution in plants. Results of the present work demonstrated that the effects of NaCl are altered by the

presence of NAs. Therefore, plant species selected for the reclamation of oil sands mining areas should be tested for their sensitivity to salts and naphthenates.

Table 6. 1. Effects of NAs on shoot and root fresh weights in NaCl-treated jack pine seedlings. Values are means ± SE, n= 6.

		Naphthenic acids	
	NaCl levels	- NAs	+ NAs
Shoot FW (g)	Control	6.12 ± 0.48	5.40 ± 0.77
	45 mM NaCl	4.57 ± 0.61	3.98 ± 0.36
Root FW (g)	Control	2.84 ± 0.29	2.86 ± 0.32
	45 mM NaCl	2.49 ± 0.28	2.55 ± 0.23

Table 6. 2. Effects of NAs on tissue Total N, K^+, Mg^{2+} and SO_4^{2-} concentrations of NaCl-treated jack pine seedlings. Values are means ± SE, n = 6. Ion concentrations are expressed in mg g^{-1} DW.

	NaCl levels	Naphthenic acids	
		- NAs	+ NAs
A. Shoot			
Total N	Control	1.68 ± 0.15	1.56 ± 0.17
	45 mM NaCl	1.67 ± 0.09	1.75 ± 0.18
K^+	Control	9.23 ± 0.63	7.97 ± 1.12
	45 mM NaCl	7.54 ± 0.96	8.02 ± 0.78
Mg^{2+}	Control	3.25 ± 0.26	3.08 ± 0.30
	45 mM NaCl	2.95 ± 0.20	3.03 ± 0.16
SO_4^{2-}	Control	4.90 ± 0.71	5.28 ± 0.88
	45 mM NaCl	5.12 ± 0.42	4.65 ± 0.63
B. Root			
Total N	Control	1.94 ± 0.30	1.76 ± 0.35
	45 mM NaCl	1.89 ± 0.09	1.75 ± 0.01
K^+	Control	3.45 ± 0.93	4.94 ± 1.88
	45 mM NaCl	2.40 ± 0.89	1.27 ± 0.80
Mg^{2+}	Control	3.47 ± 0.23	3.52 ± 0.15
	45 mM NaCl	2.93 ± 0.15	2.79 ± 0.15
SO_4^{2-}	Control	1.57 ± 0.42	2.63 ± 0.61
	45 mM NaCl	1.67 ± 0.68	3.24 ± 0.44

Table 6. 3. Effects of NAs on ionic composition, NAs concentrations and osmotic potentials of xylem sap of NaCl-treated jack pine seedlings. Values are means ± SE, n = 5 unless otherwise stated.

	NaCl levels	Naphthenic acids	
		- NAs	+ NAs
Na^+ (mg g^{-1})	Control	0.07 ± 0.00	0.08 ± 0.01
	45 mM NaCl	0.91 ± 0.02	0.94 ± 0.40
Cl^- (mg g^{-1})	Control	BDL	0.01 ± 0.01
	45 mM NaCl	1.54 ± 0.03	1.51 ± 0.06
K^+ (mg g^{-1})	Control	0.17 ± 0.02	0.15 ± 0.00
	45 mM NaCl	0.14 ± 0.02	0.20 ± 0.02
Osmotic potentials (MPa)	Control	-0.07 ±0.02	-0.17 ± 0.02
	45 mM NaCl	-0.30 ± 0.05	-0.45 ± 0.02
Naphthenic acids (mg l^{-1}); n=2	Control	-	28.60 ± 4.70
	45 mM NaCl	-	84.11 ± 16.56

BDL – Below detection level

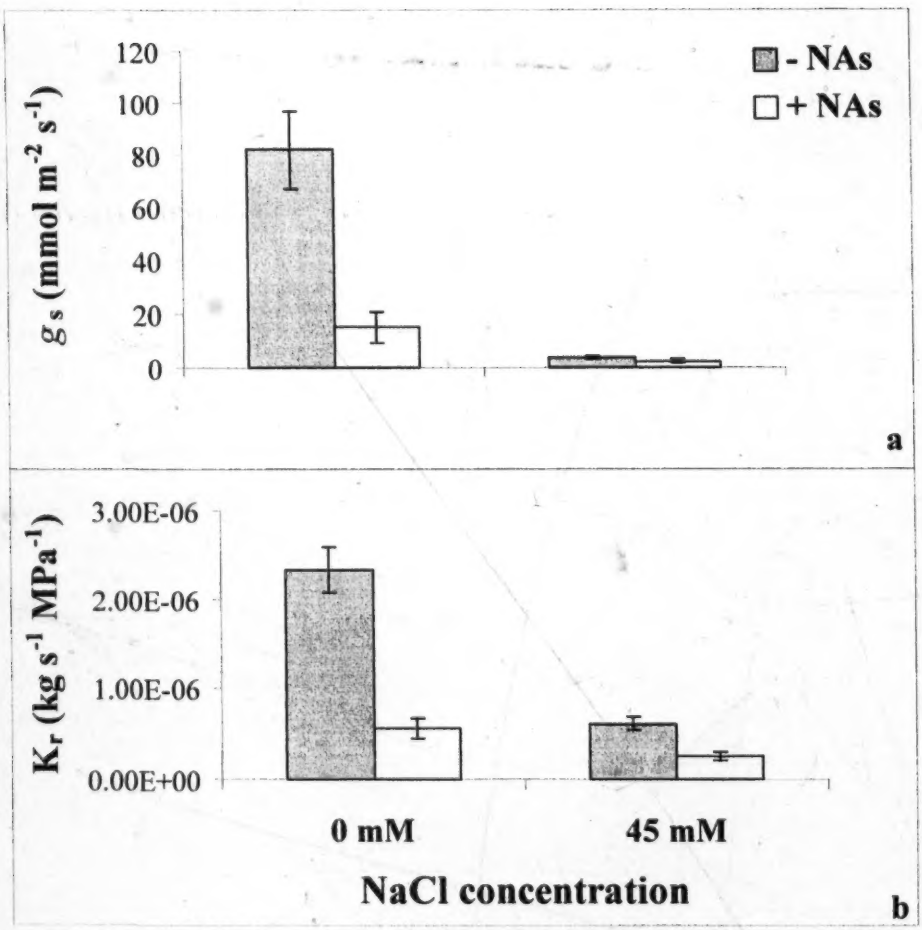

Figure 6. 1. Effects of NAs on a) stomatal conductance (g_s) and b) root hydraulic conductance (K_r) of NaCl-treated jack pine seedlings. Each data point represents mean (n=6) ± SE.

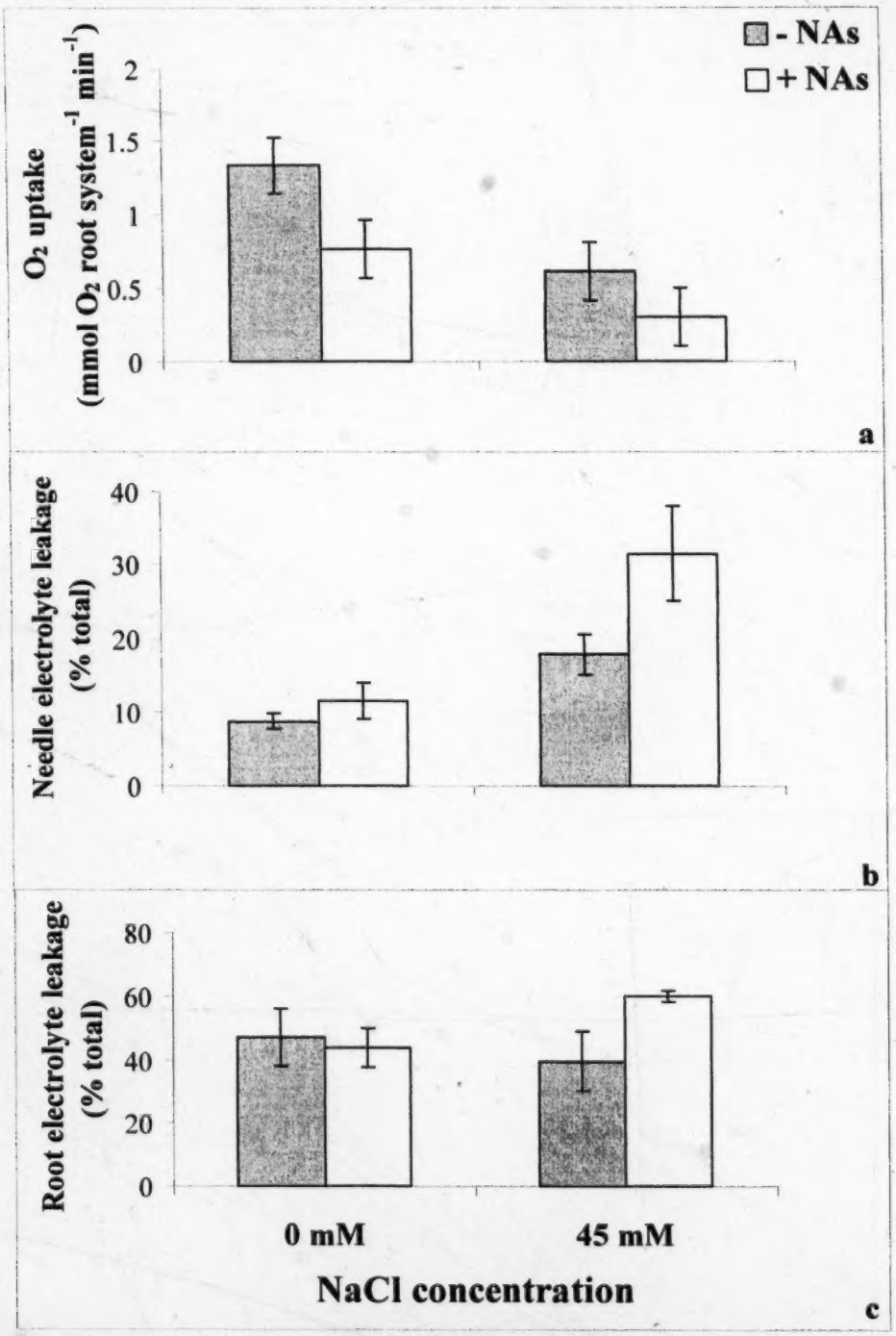

Figure 6. 2. Effects of NAs on a) root respiration, b) needle electrolyte and root electrolyte leakage of NaCl-treated jack pine seedlings. Each data point represents mean (n=6) ± SE.

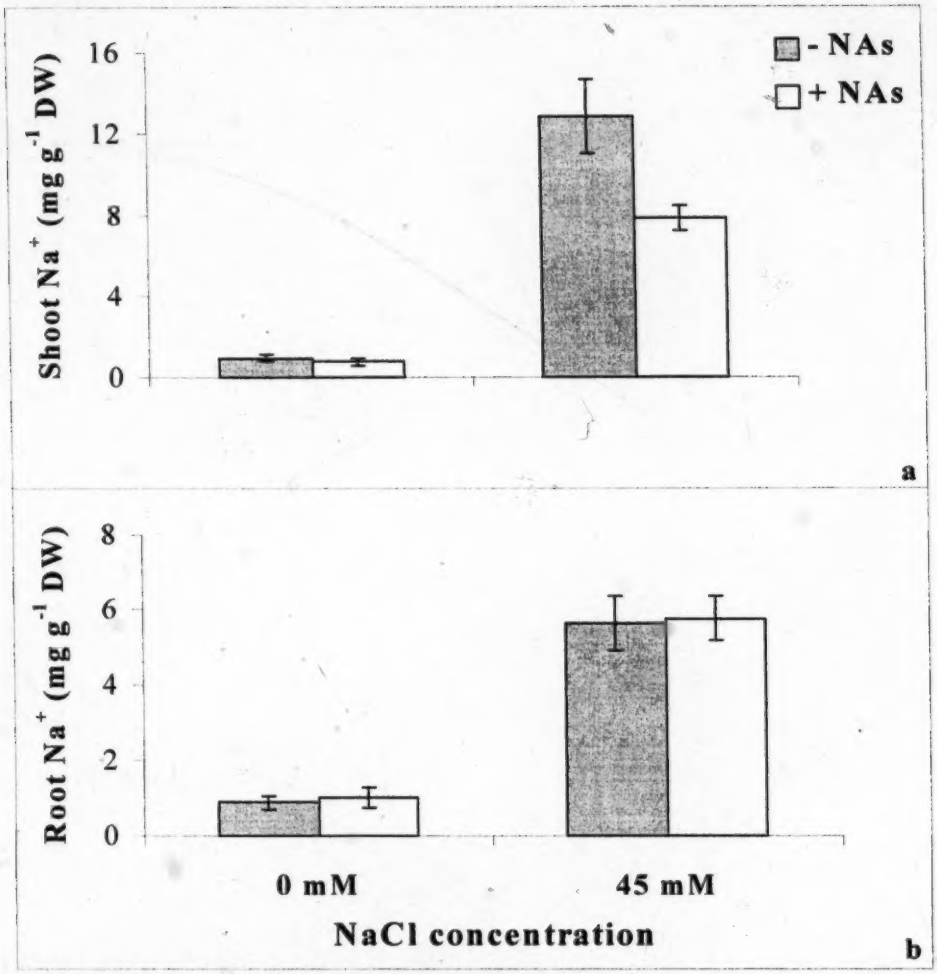

Figure 6. 3 Effects of NAs on Na^+ concentrations in a) shoots and b) roots of NaCl-treated jack pine seedlings. Each data point represents mean (n=6) ± SE.

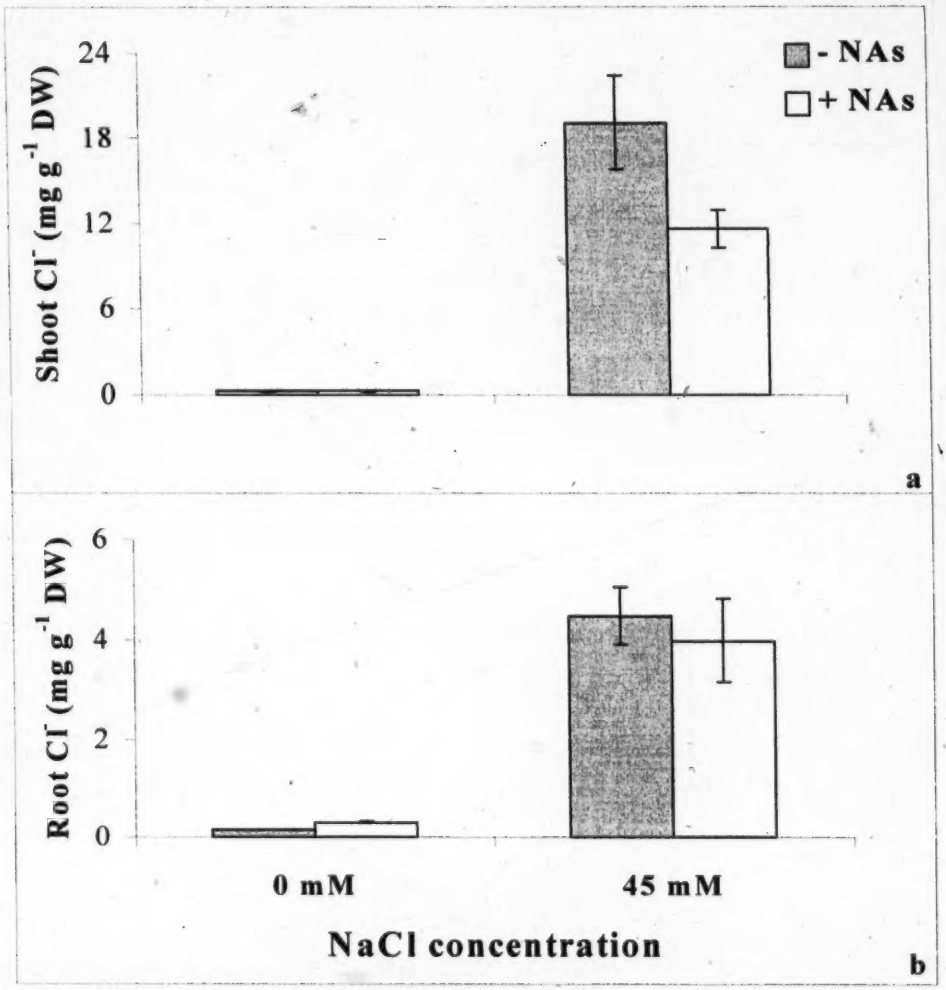

Figure 6. 4 Effects of NAs on Cl^- concentrations in a) shoots and b) roots of NaCl-treated jack pine seedlings. Each data point represents mean (n=6) ± SE.

6.5. References

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6.6 Appendices

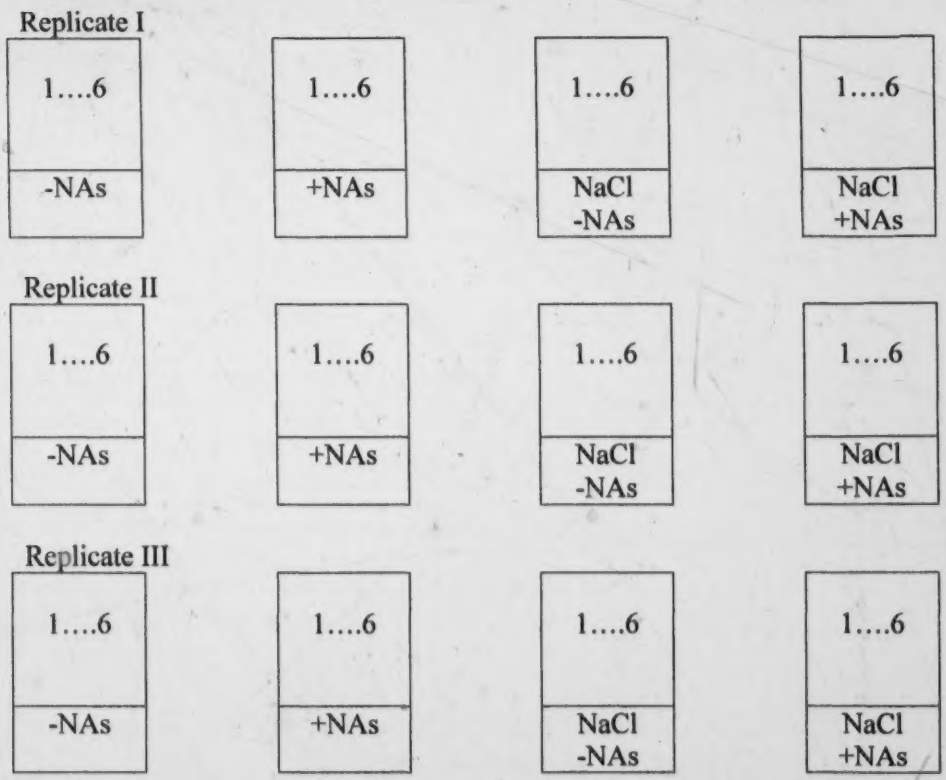

Figure A.6.1. Experimental layout of the study. Number (n=8) represents the number of seedlings placed in each treatment container, which was replicated three times

Table A.6. 1 ANOVA for tissue fresh weights, water conductance, root oxygen uptake and tissue electrolyte leakage of jack pine seedlings treated with NaCl (0 and 45 mM) and NAs (0 and 150 mg - l), with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Shoot fresh weight	NaCl	1	6.66	0.0218
	NAs	1	1.61	0.2254
	Rep	2	0.97	0.4044
	NaCl*NAs	1	0.07	0.7911
	Error	14		
Root fresh weight	NaCl	1	1.20	0.2920
	NAs	1	0.00	0.9572
	Rep	2	1.49	0.2598
	NaCl*NAs	1	0.09	0.7733
	Error	14		
Stomatal conductance (g_s)	NaCl	1	29.96	0.0001
	NAs	1	15.69	0.0014
	Rep	2	0.82	0.4614
	NaCl*NAs	1	16.29	0.0012
	Error	14		
Root hydraulic conductance (K_r)	NaCl	1	33.89	<0.001
	NAs	1	37.43	<0.001
	Rep	2	3.30	0.0629
	NaCl*NAs	1	18.62	0.0005
	Error	14		
Root oxygen uptake	NaCl	1	8.71	0.0150
	NAs	1	5.03	0.0417
	Rep	2	2.82	0.0935
	NaCl*NAs	1	0.47	0.5029
	Error	14		
Needle electrolyte leakage	NaCl	1	20.69	0.0005
	NAs	1	6.29	0.0262
	Rep	2	2.15	0.1560
	NaCl*NAs	1	2.65	0.1275
	Error	14		
Root electrolyte leakage	NaCl	1	0.31	0.5878
	NAs	1	1.31	0.2723
	Rep	2	0.90	0.4319
	NaCl*NAs	1	2.58	0.1324
	Error	14		

Table A.6. 2 ANOVA for tissue elemental concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and NAs (0 and 150 mg $^{-1}$), with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Shoot Total N	NaCl	1	2.68	0.1239
	NAs	1	0.25	0.6227
	Rep	2	1.80	0.2009
	NaCl*NAs	1	2.81	0.1156
	Error	14		
Root Total N	NaCl	1	0.00	0.9508
	NAs	1	1.80	0.2010
	Rep	2	0.60	0.5644
	NaCl*NAs	1	0.01	0.9283
	Error	14		
Shoot K^+	NaCl	1	1.17	0.2982
	NAs	1	0.12	0.7327
	Rep	2	0.59	0.5697
	NaCl*NAs	1	0.84	0.3762
	Error	14		
Root K^+	NaCl	1	5.63	0.0352
	NAs	1	0.04	0.8480
	Rep	2	1.23	0.3262
	NaCl*NAs	1	1.58	0.2324
	Error	14		
Shoot Mg^{2+}	NaCl	1	0.23	0.6389
	NAs	1	0.01	0.9237
	Rep	2	0.59	0.5698
	NaCl*NAs	1	0.21	0.6569
	Error	14		
Root Mg^{2+}	NaCl	1	12.66	0.0032
	NAs	1	0.04	0.8498
	Rep	2	0.35	0.7120
	NaCl*NAs	1	0.31	0.5879
	Error	14		
Shoot SO_4^{2-}	NaCl	1	0.07	0.7995
	NAs	1	0.04	0.8381
	Rep	2	0.77	0.4833
	NaCl*NAs	1	0.21	0.6534
	Error	14		
Root SO_4^{2-}	NaCl	1	0.18	0.6795
	NAs	1	5.02	0.0418
	Rep	2	0.37	0.6968
	NaCl*NAs	1	0.21	0.6511
	Error	14		

Table A.6. 3 ANOVA for tissue Na^+ and Cl^- concentrations of jack pine seedlings treated with NaCl (0 and 45 mM) and NAs (0 and 150 mg^{-1}), with treatment containers replicated (Rep) three times.

Parameter	SV	df	F	Pr>F
Shoot Na^+	NaCl	1	154.82	0.0001
	NAs	1	13.19	0.0034
	Rep	2	0.17	0.8474
	NaCl*NAs	1	10.82	0.0065
	Error	14		
Root Na^+	NaCl	1	83.44	0.0001
	NAs	1	0.20	0.6655
	Rep	2	2.84	0.0975
	NaCl*NAs	1	0.49	0.4975
	Error	14		
Shoot Cl^-	NaCl	1	108.28	0.0001
	NAs	1	7.26	0.0209
	Rep	2	0.31	0.7394
	NaCl*NAs	1	6.66	0.0256
	Error	14		
Root Cl^-	NaCl	1	22.36	0.0006
	NAs	1	0.35	0.5673
	Rep	2	7.13	0.0103
	NaCl*NAs	1	0.02	0.8842
	Error	14		

Table A.6. 4 ANOVA for Na^+, Cl^-, and K^+ concentrations, and osmotic potentials of xylem sap of jack pine seedlings treated with NaCl (0 and 45 mM) and NAs (0 and 150 mg $^{-1}$).

Parameter	SV	df	F	Pr>F
Na^+	NaCl	1	1633.26	<0.0001
	NAs	1	0.94	0.3468
	NaCl*NAs	1	0.17	0.6890
	Error	15		
Cl^-	NaCl	1	6595.32	<0.0001
	NAs	1	0.20	0.6639
	NaCl*NAs	1	1.66	0.2219
	Error	15		
K^+	NaCl	1	0.35	0.5626
	NAs	1	0.44	0.5162
	NaCl*NAs	1	3.44	0.0850
	Error	15		
Osmotic potential	NaCl	1	10.55	0.0070
	NAs	1	3.22	0.0979
	NaCl*NAs	1	2.16	0.1677
	Error	12		

CHAPTER SEVEN

General Discussion and Conclusions

Oil sands reclamation presents many challenges due to the presence of stress factors or plants including salinity, boron, root hypoxia and naphthenic acids (NAs) in the root zone. In the present study, I water and nutrient uptake and distribution in jack pine as influenced by boron exposure. Results of Chapter Three showed that boron reduced stomatal conductance, root water flow and caused needle injury in jack pine. Needle necrosis observed in boron-treated plants was not related to tissue nutrient deficiency.

When two or more stresses are present simultaneously, as is often the case in the oil sands reclamation sites, definitive responses are complicated (Palta 1990; Parsons 1990). Successful reclamation of oil sands following mining operations is likely to be affected by the interaction of several stress factors. Results of the previous studies suggest that salinity is among the oil sands stress factors that causes severe negative effects on plant growth (Renault et al. 1998; 1999), however, the potential synergistic effects between salts and other stress factors may be more detrimental to plants. Therefore, the general objective of the present study was to understand the mechanisms of salinity interactions with other stress factors present in the oil sands reclamation sites.

I have shown that the responses of jack pine to B were altered by salinity and that the severity of injury in jack pine varied with salinity type, with NaCl being more detrimental to jack pine than Na_2SO_4 based on needle necrosis and needle electrolyte leakage data (Chapter Four). In that study, B had only slight negative effects on growth of jack pine but when combined with salts, B decreased survival, increased needle injury

and altered tissue elemental concentrations. Since NaCl was more detrimental to plants than Na_2SO_4, as observed in Chapter Four and in previous studies (Franklin et al. 2002; Redfield and Zwiazek 2002), I used only NaCl to investigate the effects of salt and its interaction with root hypoxia in Chapter Five and NAs in Chapter Six.

A reduction in root water flow of *Populus tremuloides* has been reported to be related to root growth (Wan et al. 1999), however, root growth was not the factor which contributed to reduced flow rates in Chapter Three. The results of boron experiments (Chapter Three) revealed no direct relationship between root water uptake and root growth. On the contrary, the reduction in root hydraulic conductance observed in hypoxic NaCl-treated jack pine was partly accounted for by the observed root mortality and root growth inhibition caused by combined root hypoxia and NaCl treatments (Chapter Five). The mechanisms by which NAs and hypoxia increased resistance to root water uptake is not clear, although modifications in the root anatomy (North and Nobel 1995) and xylem cavitation, which were observed in plants exposed to environmental stresses (Byrne et al. 1977; Singh and Sale 2000), may have contributed to reduced root hydraulic conductance.

Mechanisms of salt interactions with other stress factors in jack pine are presented in Figures 7.1-3. Stress imposed by combined treatment factors, where salinity is a common factor, can be complex and seedling death may be both directly and indirectly affected by these factors. Individually, salinity and other stress factors (boron, hypoxia and NAs) inhibited stomatal conductance with a concomitant decline in root hydraulic conductance. Although the relationship between stomatal conductance and root hydraulic

conductance is not clear, Rodriguez et al. (1997) suggested that the observed reduction in root hydraulic conductance could be the cause of decline in stomatal conductance.

Reduced root hydraulic conductance and stomatal conductance appeared to be among the sensitive processes affected in jack pine by combined salinity and other stress factors. These water conductance responses appeared to be linked and, once severely inhibited they could alter root to shoot salt transport. For example, plants exposed to NAs showed lower shoot Na^+ and Cl^- concentrations than those in NaCl-treated plants (Chapter Six). This response was likely due to the negative effects of NAs on water uptake (Figure 7. 3). Theoretically, a positive relationship exists between transpiration rates and solute transport (Dalton et al. 2000). Therefore, the negative effects of NAs on water uptake led to a reduction in Na^+ and Cl^- accumulation in shoots. However, severe needle necrosis and shoot wilting were observed in NAs + NaCl-treated plants compared with plants exposed to NaCl-treatment alone, suggesting that there was a factor other than Na^+ and Cl^- accumulation in shoots contributing to upset water relations observed in NAs + NaCl-treated plants. On the other hand, results of Chapter Four showed that despite reduced transpiration rates in plants exposed to B + NaCl treatments, Na concentrations significantly increased in shoots compared to plants exposed to NaCl. It appears that factors other than water uptake govern the root to shoot Na^+ and Cl^- transport, but further experiments would be needed to verify this hypothesis.

The severity of salt stress effects on jack pine was also dependent on the nature of stress and the sites and mechanisms of stress-induced perturbations. Cellular membranes have been implicated as the sites of response of various environmental factors (Levitt 1980). Mansour (1995) and Zwiazek and Shay (1988) provide direct evidence indicating

that changes in membrane composition are correlated with alterations in membrane permeability. Excessive accumulation of Na^+ and Cl^- in the shoots is partly explained by the reduced ability of roots for Na^+ and Cl^- storage (Yeo et al. 1977; Grieve and Walker 1983). This response was likely due to the effects of NaCl on root membrane integrity. In jack pine, excessive accumulation of Na^+ could be partly explained by the alteration of cell membrane permeability which was induced by Cl^-. The severe injury observed in B + NaCl (Chapter Four) and hypoxic NaCl (Chapter Five) treated jack pine suggests that Cl^- significantly contributed to Na^+ and B toxicity primarily due to its effect on cell membrane integrity. Compositional changes in membrane phospholipids, sterols, and glycolipid components have been correlated with Cl^- accumulation under saline conditions (Kuiper 1968). Chloride affects membrane integrity leading to an increase in Na^+ uptake and accumulation in shoots (Chapter Four). Results of the present study clearly suggest that Cl^- was partly responsible for the injury observed in NaCl-treated jack pine (Figure 7.1). The results suggest that jack pine seemed to accumulate more Cl^- in tissues than Na^+, and under hypoxic conditions, roots lost the ability to restrict Cl^- uptake (Figure 7. 2, Chapter Five). Results of Chapter Six also demonstrated higher concentrations of Cl^- in shoots compared with Na^+. The high concentrations of Cl^- in the xylem sap indicate greater delivery of Cl^- to shoots than Na^+ suggesting that jack pine had lower capacity for Cl^- storage.

Plants tolerate saline conditions through ion exclusion and reduction in translocation of ions to the shoot, however, in the presence of other stress factors, these mechanisms are likely to be less or no longer effective, which results in excessive salt accumulation. There have been indications that the ability of plants to exclude salts is

related to adenosine triphosphate (ATP) levels (Barrett-Lennard 1986), which are possibly affected by environmental factors, however, the exact mode of action is not clearly understood. In Chapter Five, the lower ability of jack pine to sequester Na^+ and Cl^- could be related to the effects of hypoxia on respiration. Also, it has been reported that when seedlings failed to exclude salts from leaf tissue, the resulting effects may include water stress, ion toxicity and hormonal imbalances (Greenway and Munns 1980; Poljakoff-Mayber 1988). Some glycophytes tolerate salt by taking up salts into leaves and compartmentalizing them in vacuoles (Munns 2002) with concomitant production of organic solutes for osmotic adjustment (Yeo 1983). Although it is beyond the scope of the present study, research at the cellular level such as intra-cellular distribution of Na^+ and Cl^- and their mode of action on jack pine would help increase the understanding of the contributory roles of Na^+ and Cl^- and the main mechanism regulating salt accumulation in jack pine.

The lack of significant NA interactions with NaCl on some of the measured parameters suggests that the combined treatments may have affected those parameters before these measurements were made. This hypothesis requires further investigation. Also, the responses of jack pine to these combined stresses could be affected by factors such as seedling age, concentrations of salt used (large fluxes of Na^+ and Cl^- dominated the combined effects of stress factors) and treatment duration.

There have been reports of salinity interactions between boron and hypoxia in natural conditions and of mechanisms by which boron and hypoxia induce salt injury. To date, there have been no published reports available on NAs and salinity interactions. Therefore this area of research awaits additional work in relation to oil sands reclamation.

Since jack pine appears to be sensitive to salinity and its susceptibility to salts further increased in the presence of boron, root hypoxia and NAs, planting of jack pine is not recommended on sites where salinity and other environmental stress interactions are likely to occur together. Important salt tolerance mechanisms rely on the ability of plants to continue water uptake in the presence of high salt concentrations and other stress factors. The present work suggests that, for jack pine, stomatal control of transpiration, regulation of water and salt uptake, and specifically Cl^-, are the critical factors affecting the ability of plants to tolerate saline oil sands tailings. Still, the immediate challenge is to further understand how different stress factors influence the primary physiological processes involved in the uptake and root to shoot transport of Na^+ and Cl^-, as well as water uptake and mineral nutrition in jack pine.

Reclamation planners should formulate strategies to mitigate the effects of interacting stresses on growth and survival of potential species for reclamation. To improve survival and growth of reclamation plants, efforts should be made to alleviate salinity problem, which is the predominant limiting factor affecting plant growth in the oil sands reclamation areas. One option is to ameliorate saline soils through inoculation of seedlings with mycorrhizae. Muhsin and Zwiazek (2002) have revealed that mycorrhizae were effective in maintaining high root water flow and preventing Na^+ accumulation in *Picea glauca*. Since jack pine forms ectomycorrhizal associations, it is possible that these fungi will help the trees continue water uptake and prevent salt accumulation in tissues. Therefore, selection of mycorrhizal associations that will help jack pine improve tolerance to combined stress factors and increase survival, are worthy of further investigation.

The possible effects of numerous stress factors, though beyond the scope of this research, are likely to be important and therefore, are worthy of further study. One limitation of the present study is that the experiments were conducted under controlled environmental conditions, which did not reflect the temporal and seasonal fluctuations of environmental factors present in the reclamation areas. Therefore, results of the present study, similar to those of any other study conducted in controlled environmental conditions, require careful evaluation before they can be translated into practical recommendations. The present study was intended to provide a basis of reference and the results should only be considered indicative of possible responses of jack pine under field conditions. However, the information obtained from the present work can help reclamation efforts by providing the baseline information concerning jack pine responses to salinity and the interactions between salinity, boron, root hypoxia and naphthenic acids.

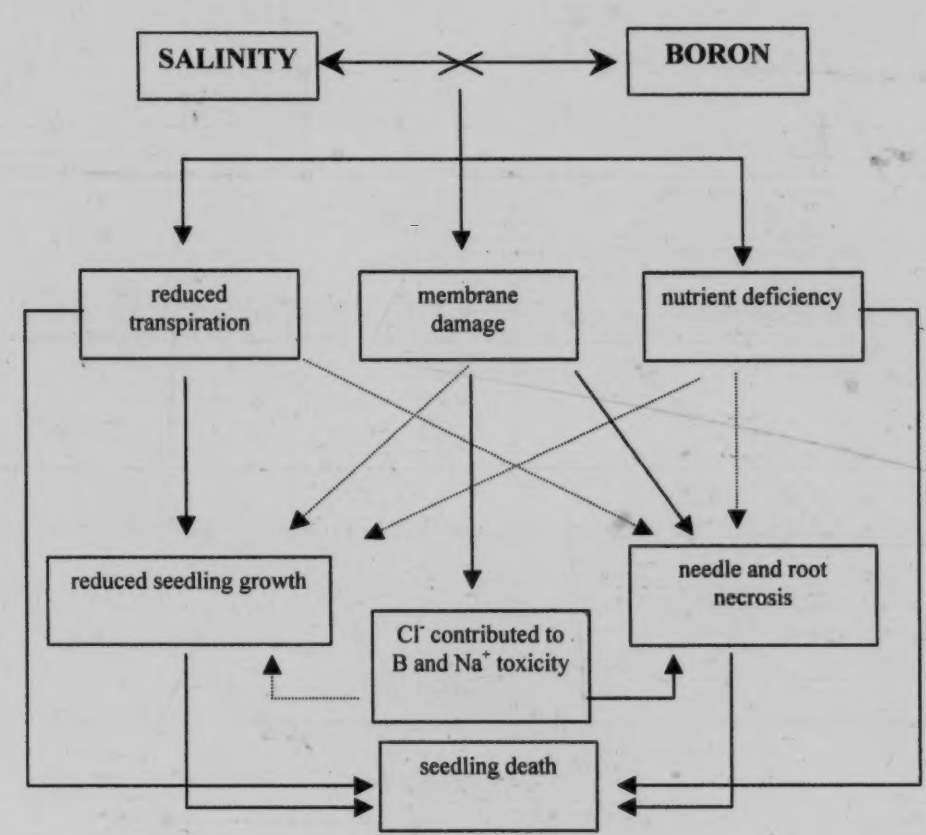

Figure 7. 1. Model of salinity and B interactions in jack pine (*Pinus banksiana*) seedlings. Broken lines indicate that relationship between responses is poorly understood.

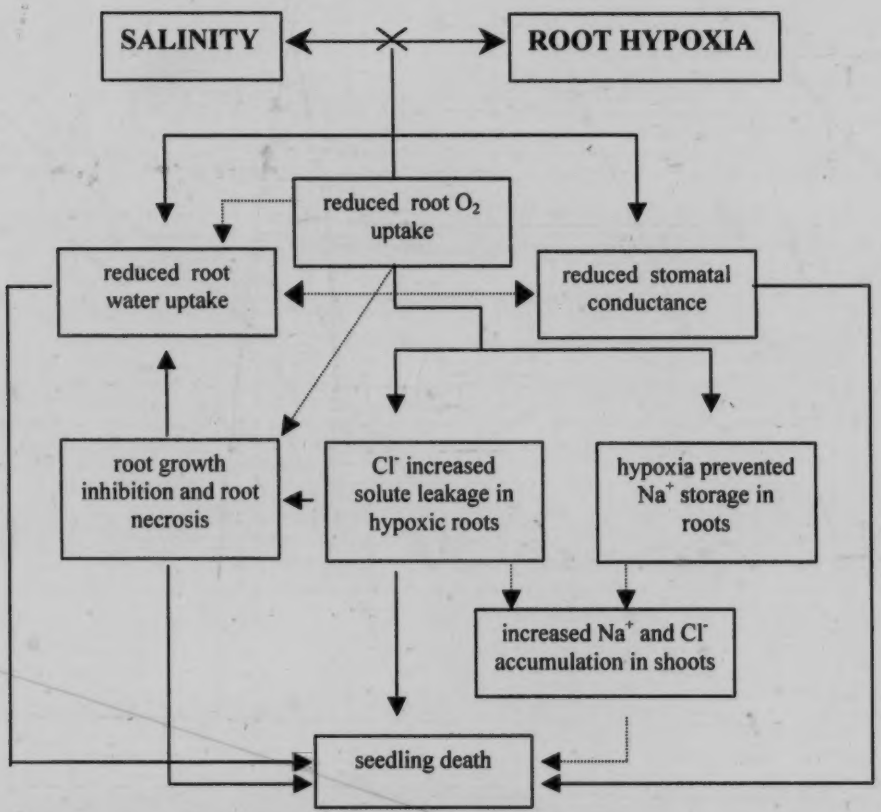
SALINITY
ROOT HYPOXIA
reduced root O_2 uptake
reduced root water uptake
reduced stomatal conductance
root growth inhibition and root necrosis
Cl^- increased solute leakage in hypoxic roots
hypoxia prevented Na^+ storage in roots
increased Na^+ and Cl^- accumulation in shoots
seedling death

Figure 7. 2. Model of salinity interactions with root hypoxia in jack pine (*Pinus banksiana*) seedlings. Broken lines indicate that relationship between responses is poorly understood.

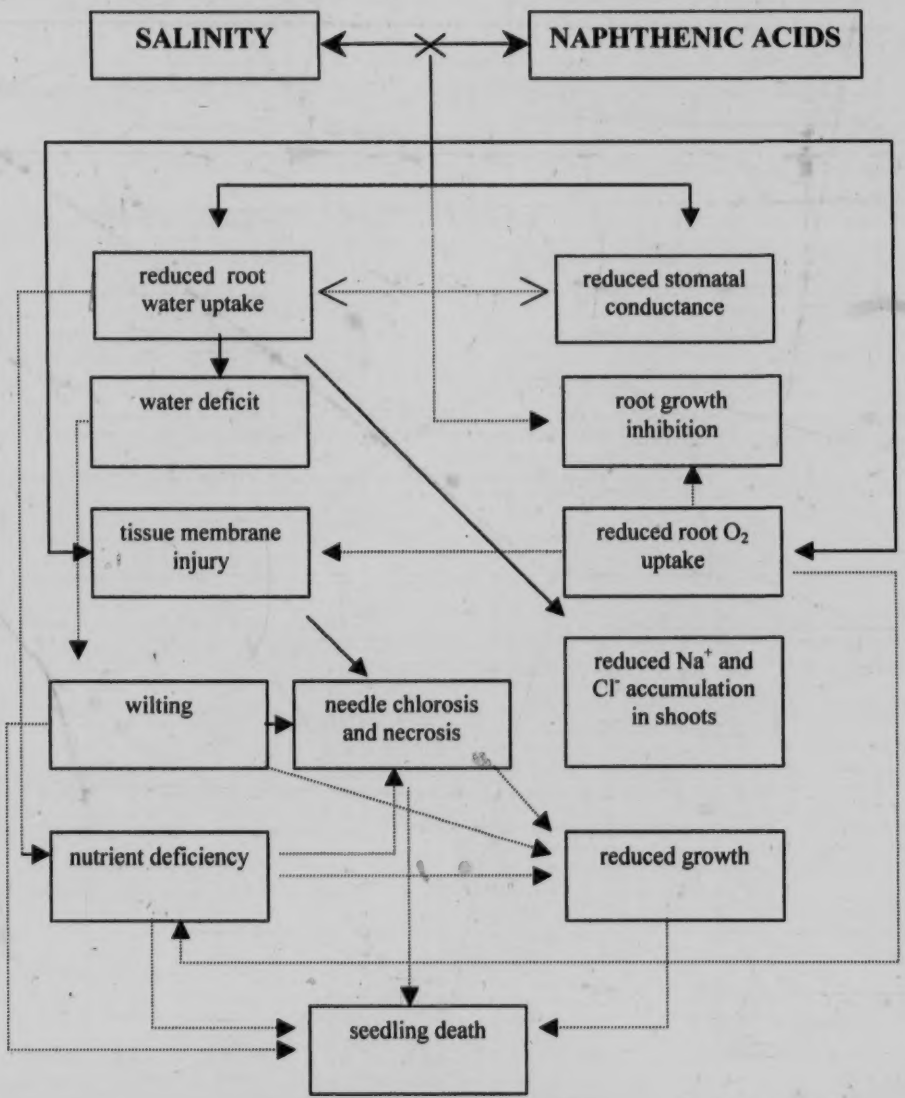

Figure 7. 3. Model of salinity interactions with NAs in jack pine (*Pinus banksiana*) seedlings. Broken lines indicate that relationship between responses is poorly understood.

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